

### REMARKS

Claims 1-41 are pending in the application. Claims 1-3, 19, 21 and 22 are rejected as related to the species under consideration, that is, I (a), the method wherein the absence of effective CD+4 T cell help is attained by exclusion of CD+4 T cells, and the species of II (a), tumor antigens. It is Applicants' understanding that claims 1, 2, 4, 15-19, 21 and 22 remain under consideration as related to the subject matter of the elected species I (d), a method wherein the absence of effective CD+4 T cell help is attained by inhibiting signaling consequences to dendritic cell T-cell engagement and species II (b), viral antigens. The Examiner has noted that the previous rejections have been mooted in light of the amendments as filed. Accordingly, claims 1-4, 15-19, 21 and 22 remain under consideration.

Claims 1, 2, 3, 19, 21 and 22 were rejected under 35 U.S.C. §112, first paragraph as failing to comply with the enablement requirement. Applicants respectfully traverse the Examiner's rejection, and have also provided support in the specification for enablement of the invention as originally filed. Support for this can be found in the application on pages 13, lines 18-23 continuing on to page 14, lines 1-13; and on page 25, lines 3-16, and further on page 32, Example 2, in particular on page 35, lines 20-24, continuing on to page 36, lines 1-23 and continuing on to page 37, lines 1-3. Additional support can be found in the attached reference articles (Exhibits A and B1-B3), attached herein for the convenience of the Examiner. Thus, withdrawal of the rejection under 35 U.S.C. §112, first paragraph, is respectfully requested.

#### ***Claim Rejections under 35 U.S.C. §112***

Claims 1, 2, 3, 19, 21 and 22 have been rejected under 35 U.S.C. §112, first paragraph for failing to comply with the enablement requirement. Applicants respectfully traverse the Examiner's rejection, and have also provided support for enablement of the invention as claimed in the specification on page 13, lines 18-23 continuing on to page 14, lines 1-13; and on page 25, lines 3-16, and further on page 32, Example 2. In particular, page 35, lines 20-24, continuing on to page 36, lines 1-23 and continuing on to

page 37, lines 1-3, distinctly point out how to measure apoptosis in CD+8 T cells using procedures known to those skilled in the art. In particular, following specified culture conditions for 7 days with dendritic cells as outlined in the present application, T cells were stained with a fluorescent dye, CFSE, to track cellular division. Afterwards, apoptosis was measured using a stain specific for cells undergoing apoptosis, Annexin V.

While it is recognized that the model utilized to teach the novel methods for inducing tolerance to specific antigens in the instant application is a viral system, Applicants respectfully point out to the Examiner that induction of a T cell response to either viral or tumor antigens occurs via a common pathway that is known to those skilled in the art. That is, it is recognized that induction of a T cell response to either viral or tumor antigens occurs via presentation of the antigens in the context of MHC molecules and in the presence of CD+4 T helper cells and co-stimulatory molecules.

Given these facts, in conjunction with the methods presented in the instant application, that is, presenting the antigen (which may be viral, tumor, self antigens or transplantation antigens) to the T cell by way of the dendritic cell in the presence of a maturation stimulus, but in the absence of CD+4 T cell help, Applicants assert that tolerance would result irrespective of the antigen employed. Further, as noted on page 35, lines 20-23:

“One possibility is that the proliferating cells were being deleted, thus accounting for the *in vivo* phenomenon of cross-tolerance (C. Kurts et al., J Exp Med 186, 2057-62, 1997). To directly test this possibility, an assay was established to detect T cell apoptosis while tracking the number of cell divisions.”

The deletion of the T cells via the apoptotic pathway was evident as noted in the instant application on page 36, lines 13-17 and in Figure 6. Applicants note:

“Using FACS analysis, the HLA-DR<sup>+</sup> T cells were gated, and simultaneously evaluated for their CFSE fluorescence and Annexin V staining. On day 3, 12% of the HLA-DR<sup>+</sup>, CD8<sup>+</sup> T cells had divided and initiated an apoptotic pathway. On day 5, 38% of the dividing HLA-DR<sup>+</sup>, CD8<sup>+</sup> T cells were Annexin V<sup>+</sup>. And by day 7, 55% of the proliferating HLA-DR<sup>+</sup>, CD8<sup>+</sup> T cells had committed to die (**Figure 6**).”

Applicants have also provided support in the literature for the rationale as to why one may want to induce tolerance to tumor cell antigens. In particular, Applicants have

provided a copy of several reference articles, one of which is entitled "The Risk of Autoimmunity Associated with Tumor Immunotherapy" by Eli Gilboa (2001) Nature Immunology Vol. 2: 789-792, attached herein as Exhibit A.

The Examiner asserts that the invention is drawn to a method for inducing tolerance to a tumor antigen in a mammal, and that the art teaches that many patients have tolerance to their own tumor antigens. Furthermore, the Examiner asserts that the art teaches that it is desirable to break this tolerance to allow for an anti-tumor immune response. Yet further, the Examiner asserts that there are no teachings in the art or in the specification on the benefits of inducing apoptosis in tumor specific CD+8 T cells; since in so doing one would eliminate tumor specific CD+8 cells capable of destroying tumor cells, which would result in a therapeutic response. Applicant respectfully points out to the examiner that the claims as currently pending are drawn to an *ex vivo* system whereby one can control the type of response that one desires followed by transfer of the desired cellular population to the patient in need of such therapy.

Support in the specification for inducing tolerance to tumor antigens can be found on page 7, lines 4-6, wherein it is stated that:

"Although current immunotherapy strategies to treat tumors are aimed at activating tumor-specific T cells, in some instances, autoimmunity has occurred. At such times, it would be useful to have strategies to interrupt this aberrant immune attack."

Furthermore, it is also stated on page 7, lines 10-12:

"Thus, suitable antigens for which tolerance is desirably induced by the methods of the invention include but are not limited to self antigens, transplant antigens, tumor antigens, and viral antigens, but these are merely illustrative and non-limiting."

Thus, it is with respect to induction of tolerance to specific antigens (using an *ex vivo* system), of which tumor antigens are a non-limiting example, using the methods described in the present application, that the claims are directed. Accordingly, further support for such a strategy may be found in the accompanying article attached herein as Exhibit A by Eli Gilboa, wherein vaccination of subjects with tumor cell antigens is discussed and wherein a discussion of the risks involved in such an approach is provided.

Gilboa states on page 791, first paragraph, that:

“Because the identification and isolation of unique tumor antigens from each cancer patient is currently not an option, the alternative would be to vaccinate with tumor-derived antigenic mixtures isolated from each patient. If vaccination with nonmutated shared antigens, despite their reduced potency, proves therapeutically effective, common “off-the-shelf” reagents will be available for treating many cancer patients. But what are the risks of autoimmunity associated with each approach?”

Furthermore, on page 791, in paragraph 2:

“It is important to appreciate that in the induction of autoimmune pathology, the circumstances that surround the stimulation of anti-self responses during pathogen infection differ significantly from the circumstances that prevail during tumor vaccination with self-antigens. During pathogen replication, activation of autoreactive T cells has evolved to be limited so that it causes minimal damage with no physiological consequences. In contrast, effective and repeated vaccinations with selected self-antigens will be capable of activating large numbers of autoreactive T cells, including high-avidity “ignored” T cells as well as low-avidity T cell “escapees”, **and hence will generate powerful autoimmune responses that are greater than those that the system has evolved to tolerate.** This will increase considerably the risk of autoimmune pathology, as has been vividly shown in animal studies. Skin depigmentation (vitiligo), prostatitis, experimental autoimmune encephalomyelitis (EAE), diabetes and cardiomyopathy were all induced in mice vaccinated with tissue-specific self-antigens.”

Yet further, on page 791, paragraph 3:

“Tumor-specific antigens make up only a small fraction of the total antigen isolated from tumor cells. It is, therefore, not surprising that it is commonly argued that effective vaccination protocols with unfractionated mixtures of tumor-derived antigens—in contrast to vaccination with a single self-tumor antigen—carries a heightened risk of destroying tolerance of self-antigen.”

Further support for enablement can be found in the accompanying articles by the inventors of the present application, attached herein for the Examiner’s convenience as Exhibits B1, B2 and B3.

In particular, the articles entitled “Tumor specific killer T cells in paraneoplastic cerebellar degeneration” (attached herein as Appendix B1), “Paraneoplastic syndromes involving the nervous system” (attached herein as Appendix B2), and “Detection and treatment of activated T cells in the cerebrospinal fluid of patients with paraneoplastic cerebellar degeneration” (attached herein as Appendix B3), clearly point out that while it is possible to measure a tumor specific cytolytic T cell response in the patients described,

it is also evident that a paraneoplastic neurological disorder is associated with tumor cell expression of neuron specific proteins. Accordingly, the cytolytic T cells were induced by the tumor cell expression of a protein normally expressed in immune-privileged tissues. It is likely then that the cytolytic T cells that were induced contributed to the neuronal degeneration observed in these patients. Furthermore, articles B2 and B3 clearly point out that tumor immunity can co-exist with an autoimmune disease. Thus, there is a need to better control the immune response to tumor cell antigens, for example, by way of the methods described in the present application.

Based on the support for enablement of the claims as drawn to tumor antigens provided herewith, withdrawal of the rejection is thus respectfully requested.

***Fees***

No fees are believed to be necessitated by the instant response. However, should this be in error, authorization is hereby given to charge Deposit Account No. 11-1153 for any underpayment, or to credit any overpayments.

***Conclusion***

Withdrawal of the rejections is respectfully requested. If a discussion with the undersigned will be of assistance in resolving any remaining issues, the Examiner is invited to telephone the undersigned at (201) 487-5800, ext. 118, to effect a resolution.

Respectfully submitted,



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Attachments: Appendix A, B1, B2, B3

## APPENDIX A

The development of increasingly powerful methods to stimulate anti-tumor immune responses carries the risk of breaking tolerance to self and causing autoimmune pathology. How concerned should we be?

# The risk of autoimmunity associated with tumor immunotherapy

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The theory that evolution has devised simple and optimal solutions to the multiple challenges presented by the regulation of an immune system has largely been abandoned. For example, to solve the problem of discrimination between self and non-self, the immune system has developed a patchwork of imperfect solutions that are far from fail-safe. Autoimmunity is, in fact, an intrinsic feature of the immune response to infectious agents; however, effective checks and balances are in place so that autoimmunity rarely proceeds to autoimmune disease. Increasingly powerful immunological interventions that target self-antigens—inadvertently or by design—could potentially upset this delicate balance and destroy self-tolerance. This commentary will examine the autoimmune risks associated with tumor immunotherapy in light of our current, and still emerging, understanding of immunological tolerance to self.

## The basis of tolerance to self-antigens

Naïve T cells do not circulate throughout the body in search of foreign antigens, instead they are restricted to hematopoietic and lymphoid tissues; for this reason they do not come into contact with antigens in peripheral tissues<sup>1</sup>. Antigens are channeled to the naïve T cells via a network of professional antigen-presenting cells (APCs) that consists of bone marrow-derived dendritic cells (DCs). In response to infection, the DCs undergo a differentiation process called maturation; during this process they up-regulate the capacity to present captured antigens and then migrate to draining lymph nodes where they encounter and activate cognate T cells<sup>2</sup>. Because DCs do not discriminate between the antigens they capture at the site of inflammation, the prerequisite maturation of DCs for the activation of naïve T cells has evolved to favor the generation of immune responses to foreign pathogen-specific antigens. Discrimination is, however, far from complete because the maturing DCs will present self-antigens along with pathogen-specific antigens released from the dying cells. Self-antigens will be also presented alongside tumor-specific antigens during an anti-tumor immune response. Thus, the question is whether or how can we stimulate an effective anti-tumor response without simultaneously inducing autoimmunity. To do that, we have to understand how autoimmunity is avoided during a normal immune response; we also have to determine what is required in order to stimulate an effective anti-tumor response in cancer patients who normally do not respond immunologically to their tumors.

Autoreactive T cells are eliminated in the thymus and in the periphery when presented with self-antigen and stimulated via the T cell receptor (TCR). Elimination of autoreactive T cells is, however, incomplete. T cells with high avidity are preferentially eliminated, whereas low-avidity T cells escape deletion and are retained in the mature naïve T cell population<sup>3-6</sup>. Because the repertoire of available

T cells exhibits a range of avidities to a particular major histocompatibility complex (MHC)-peptide combination, presentation of a high density of MHC-peptide will cause the elimination of the higher avidity T cells. Expression of costimulatory molecules on the APC would further reduce the pool of antigen-specific T cells with high avidity. It is conceivable that both central and peripheral negative selection systems were evolutionarily "calibrated" so that somatic cells would not be capable of activating the remaining autoreactive T cells with low avidity, during homeostasis as well as during common pathogenic infections. One important implication of this is that the remaining low-avidity T cells are functionally competent. They can be activated by cells expressing higher densities of MHC-peptide and/or costimulatory molecules compared to the DCs that serve as APCs during negative selection in the thymus or the APCs that tolerize autoreactive T cells in the periphery (see below). Not surprisingly, cytolytic T lymphocytes (CTLs) specific to ubiquitous proteins such as  $\beta_2$ -microglobulin, hemoglobin<sup>7</sup> or kallikrein<sup>7</sup> persist in the peripheral repertoire and can be activated under appropriate conditions. Appreciating this point is key to any discussion on how to induce effective anti-tumor immunity in cancer patients and the risks of associated autoimmunity.

Autoreactive T cells that have escaped thymic deletion are comprised of low-avidity T cells that have not received a death signal of sufficient strength in the thymus and low- to high-avidity T cells that recognize cell-associated tissue-specific antigens that were not presented in the thymus. To deal with such peripheral autoreactive T cells, the immune system does one of two things: it ignores them or inactivates them (Table 1). Certain autoreactive T cells are ignored<sup>8</sup>. One reason for this is that naïve T cells are not activated in the absence of costimulation, which somatic cells lack. A second reason is that naïve T cells have no access to most antigens expressed in somatic tissues because their circulation is largely restricted to the blood and lymphoid tissues<sup>1</sup>. The phenomenon of immunological ignorance has been documented in several experimental systems<sup>9-11</sup> and likely represents one solution to the maintenance of peripheral tolerance that applies to some, but not all, autoreactive T cells. In fact, there is compelling evidence that during homeostasis—that is, in the absence of infection—active tolerance mechanisms are in place that prevent the activation of autoreactive T cells in the periphery and induction of autoimmune pathology. This is best illustrated by the fact that mice that are deficient in interleukin 2 (IL-2), cytotoxic T cell antigen-4 (CTLA-4), Fas or Fas ligand show devastating autoimmune pathology. The immune system, at least in the mouse, employs two strategies to deal with such dangerous autoreactive T cells: these are APC-based and T cell-based strategies.

A subset of immature-like DCs that present antigen to T cells in a tolerogenic manner have been characterized in mice. The tolerizing

## COMMENTARY

**Table 1. Fate of autoreactive T cells in the mature population during homeostasis, pathogen infection and vaccination with self-tumor antigens**

	Low-avidity T cells <sup>a</sup>		High-avidity T cells <sup>b</sup>	
Homeostasis	Ignored <sup>d</sup>	Eliminated <sup>d</sup>	Ignored	
Pathogen infection	Ignored <sup>d</sup>		Activated ↓ Eliminated <sup>e</sup>	
Vaccination with self-tumor antigens	Activated ↓ Eliminated <sup>d</sup> or autoimmunity <sup>f</sup>		Activated ↓ Eliminated <sup>d</sup> or autoimmunity <sup>f</sup>	

<sup>a</sup>Subthreshold presentation by thymic DCs. <sup>b</sup>Tissue-specific nonsecreted products not presented to thymic DCs. <sup>c</sup>The assumption is that both central and peripheral tolerance processes were calibrated in a manner that meant that somatic cells would not be capable of activating the remaining low-avidity autoreactive T cells during homeostasis or during common pathogenic infections. <sup>d</sup>Elimination by tolerizing APCs or regulatory T cells. <sup>e</sup>CD8<sup>+</sup> T cells eliminated by AICD after repeated presentation of antigen to somatic cells in the absence of costimulation; CD4<sup>+</sup> T cells "die by neglect" (PCD) because somatic cells do not express MHC class II. <sup>f</sup>Determined by the nature of self-antigen and the intensity of vaccination.

APCs constantly acquire self-antigens from somatic cells and home to draining lymph nodes where they present antigen to autoreactive T cells<sup>12</sup>. The function of the APC-mediated tolerance system is, therefore, to remove autoreactive T cells from the circulation to prevent their activation in the event of an infection. Conceivably, this process evolved to function primarily in tissues that are frequently the targets of pathogenic infections and consequently present self-antigens. However, the APC-mediated inactivation of autoreactive T cells is not fail-safe at preventing the activation of autoreactive T cells and induction of autoimmune pathology. In the mouse, autoreactive T cells can be, and probably constantly are, activated by fully competent APCs and would induce autoimmune pathology except for the presence of regulatory T (T<sub>R</sub>) cells. A thoroughly studied subset of such T<sub>R</sub> cells are the CD4<sup>+</sup>CD25<sup>+</sup> subset<sup>13</sup>. These T<sub>R</sub> cells prevent the activation, expansion and function of autoreactive T cells present in the mature T cell population, as shown by the fact that depletion of this subset induces a broad range of autoimmune pathologies in mice. Another subset of CD4<sup>+</sup> T<sub>R</sub> cells, which may be identical to the CD4<sup>+</sup>CD25<sup>+</sup> subset, was discovered in the course of murine transplantation studies when mice were treated with nondepleting antibodies to CD4<sup>14</sup>. The current view is that regulatory T cells fulfill an important sentinel function: they purge the body of autoreactive T cells that may have escaped elimination by the tolerizing APCs.

Thus, multiple mechanisms act in concert to eliminate autoreactive T cells or keep them in check; each process has evolved in response to a specific need and is targeted to distinct subsets of self-antigens. What we don't know at the moment is how important each process is. What proportion, and which subsets of, autoreactive T cells are ignored, tolerized by APCs or "regulated" by T cells?

### Preventing autoimmune pathology

Pathogens trigger an adaptive inflammatory response, which leads to the local differentiation of DCs into potent APCs and the activation of cognate T cells at the draining lymph node. DCs acquire antigens from the cell debris generated during the inflammatory response but do not discriminate between self and pathogen-specific antigens. Thus, autoreactive naïve T cells present at the local inflammatory site could be activated by APCs generated at the site. If this is so, why are we still here to tell the story? Low-avidity autoreactive T cells that have escaped elimination in the thymus are not expected to recognize their targets in the periphery, which include activated DCs that present

the cognate self-antigen. The burning question is how are the high-avidity autoreactive T cells that recognize antigens not presented in the thymus kept in check? The immune system appears to have evolved two complementary strategies: a defensive strategy whereby autoreactive T cells are eliminated before they can do harm and a reactive strategy whereby an autoimmune response is down-regulated before it causes too much damage.

T cells are constantly eliminated from the repertoire of mature T cells by tolerizing APCs and T<sub>R</sub> cells (defensive strategy). There is, however, no evidence that the tolerizing APC or T<sub>R</sub> cell systems can effectively eliminate all potentially autoreactive T cells. Autoreactive T cells that have escaped tolerance will be activated at the inflammatory

site, which could lead to autoimmune damage. The main strategy for containing the potential harm caused by their activation is to limit the expansion and survival of activated autoreactive T cells (reactive strategy). The activated CD4<sup>+</sup> T cells will "die by neglect" (also known as programmed cell death or PCD) because most somatic cells do not express MHC class II molecules, whereas CD8<sup>+</sup> T cells that repeatedly encounter self-antigen on somatic cells in the absence of costimulation will die in a process known as activation-induced cell death (AICD). The excess of somatic cells presenting self-antigens compared to cells presenting pathogen-specific antigens will ensure that the autoimmune damage will be limited and of no pathological consequence<sup>15</sup>.

### Tumor immunotherapy and risks of autoimmunity

There are two main reasons why naturally progressing tumors do not stimulate an effective immune response. First, tumors have evolved to grow in a manner that does not trigger an effective inflammatory response, thereby limiting the access of tumor-derived antigens to the professional APC system. Simply put, a growing tumor that does not trigger an inflammatory response is viewed by the immune system as normal tissue and subject to the same rules of tolerance. Second, tumor progression in cancer patients is often associated with the secretion of immunosuppressive factors or down-regulation of the various components of the MHC class I presentation pathway (incidentally providing circumstantial, though compelling, evidence for the "footprints" of an effective immune response that the tumors had to develop means to circumvent). In effect, most tumors are capable of—and often do trigger—a weak immune response that goes undetected and, therefore, gives rise to the misguided theory that such tumors are "nonimmunogenic". The goal of tumor vaccination is to tilt the balance in favor of tumor immunity by channeling tumor antigens into the professional APC pathway in order to more efficiently activate tumor-specific T cells.

Unique tumor antigens have been identified and characterized in both mice and humans. The majority of unique tumor antigens arise as a result of somatic mutations in normal gene products, which conceivably reflects the increased genetic instability of cancer cells. Thus unique tumor antigens can be expected to trigger neither tolerance nor autoimmunity and should, therefore, make effective targets for cancer vaccination. Shared tumor antigens—which represent the majority of tumor antigens from cancer patients that have been characterized to date—correspond for the most part to normal, nonmutated, gene products

(self-antigens) that are overexpressed, preferentially expressed or re-expressed in cancer cells. Self-tumor antigens represent a spectrum of antigens that range from antigens that have been completely ignored by the immune system to antigens that have triggered varying degrees of tolerance. The usefulness of such antigens in cancer vaccination will vary accordingly<sup>16</sup>. Because the identification and isolation of unique tumor antigens from each cancer patient is currently not an option, the alternative would be to vaccinate with tumor-derived antigenic mixtures isolated from each patient. If vaccination with nonmutated shared antigens, despite their reduced potency, proves therapeutically effective, common "off-the-shelf" reagents will be available for treating many cancer patients. But what are the risks of autoimmunity associated with each approach?

As discussed above, tolerance to autoreactive T cells is incomplete. High-avidity T cells, which correspond to self-antigens that were "ignored" by the immune system, and T cells with reduced avidity, which escaped central and peripheral tolerance, will be available for activation and tumor recognition. Thus, the avidity of anti-tumor T cells that recognize self-antigens could vary from high to low, depending on the antigen chosen for vaccination. It is important to appreciate that in the induction of autoimmune pathology, the circumstances that surround the stimulation of anti-self responses during pathogen infection differ significantly from the circumstances that prevail during tumor vaccination with self-antigens. During pathogen replication, activation of autoreactive T cells has evolved to be limited so that it causes minimal damage with no physiological consequences. In contrast, effective and repeated vaccinations with selected self-antigens will be capable of activating large numbers of autoreactive T cells, including high-avidity "ignored" T cells as well as low-avidity T cell "escapees", and hence will generate powerful autoimmune responses that are greater than those that the system has evolved to tolerate (Table 1). This will increase considerably the risk of autoimmune pathology, as has been vividly shown in animal studies. Skin depigmentation (vitilago)<sup>17,18</sup>, prostatitis<sup>19</sup>, experimental autoimmune encephalomyelitis (EAE)<sup>20</sup>, diabetes and cardiomyopathy<sup>11</sup> were all induced in mice vaccinated with tissue-specific self-antigens.

Tumor-specific antigens make up only a small fraction of the total antigen isolated from tumor cells. It is, therefore, not surprising that it is commonly argued that effective vaccination protocols with unfractionated mixtures of tumor-derived antigens—in contrast to vaccination with a single self-tumor antigen—carries a heightened risk of destroying tolerance of self-antigen. However, the phenomenon of immunodominance has taught us that antigens vary in their ability to stimulate the immune response. Because immunodominance is proportional to the frequency of cognate T cells available for activation, self-antigens—which have triggered tolerance to varying degrees—should be underrepresented in a tumor-specific T cell population that has been stimulated with unfractionated mixtures of tumor-derived antigens. Consistent with this expectation, animal studies have provided ample evidence that vaccination protocols with tumor-antigenic mixtures can stimulate potent anti-tumor immunity in the absence of detectable autoimmunity<sup>21–23</sup>. An exception, however, was a study in which mild vitilago was seen in animals treated with a combination of granulocyte macrophage-colony-stimulating factor-expressing irradiated melanoma cells and anti-CTLA-4<sup>24</sup>.

A note of caution should be sounded, however. A small number of patients with gynecological (breast and ovarian) tumors exhibit paraneoplastic neurological disorders (PNDs). Recent studies have implicated a CTL response directed against cdr2, a Purkinje neuronal-specific protein widely expressed in breast and ovarian tumors, as a con-

tributing factor in PND<sup>25</sup>. These observations suggest that in a small proportion of breast and ovarian cancer patients a naturally occurring immune response, which is directed against a tissue-specific protein that is re-expressed in cancer cells, leads to autoimmune pathology. The implications of these findings, which could also apply to other cancers, are that increasingly powerful and effective vaccination protocols that use tumor-derived antigenic mixtures could lead to an increase in the incidence of PND in patients with breast and ovarian cancers. In summary, the PCD example notwithstanding, contrary to popular belief, vaccination with patient-specific tumor-derived antigenic mixtures may not only be more effective than vaccination with single shared self-tumor antigens as some argue<sup>16,26</sup>, but also entail a reduced risk for autoimmunity.

Vaccination with specific self-antigens is, however, the real challenge. How can we stimulate an effective immune response that targets specific self-antigens expressed in tumor cells that will eradicate or contain tumor growth, without devastating autoimmune consequences. Can it be done? We will know the answer for certain once powerful T cell-based vaccination strategies have been developed; we may not have to wait long for the answer. But in the meantime, existing evidence suggests that it can be achieved. Tumors and normal tissues exhibit a differential susceptibility to immune effector arms. This may be the single most important, and largely overlooked, factor in the claim that autoimmunity may be less of an issue than many suspect. It is commonly thought that a normal cell and a tumor cell are equally susceptible to the effector immune response. For example, all things being equal, a CTL should kill a normal cell with the same efficiency that it would kill a tumor cell expressing the same antigen. This may indeed be the case when isolated cells are considered, but apparently is not the case when structured normal tissues or a tumor mass are the targets of an immune response *in vivo*.

Experimental data speak the loudest. A host of animal studies have shown that an activated effector arm that consists of (self) antigen-specific CD8<sup>+</sup> or CD4<sup>+</sup> T cells can selectively eradicate tumors in mice without apparently damaging normal tissues that are expressing the same antigen<sup>4,27–31</sup>. However, experimental data is not always quite so unequivocal: in studies in which mice were vaccinated with melanocyte-specific antigens, skin depigmentation (vitilago) accompanied tumor regression in some cases<sup>17,18</sup>, but not in others<sup>27,30</sup>, and prostatitis was seen in mice vaccinated against a prostate tissue-specific product<sup>19</sup>. These autoimmune manifestations were mild, but could reflect the fact that destruction of melanocytes or prostate tissue can be well tolerated; in other instances a similar level of autoimmunity could have more serious consequences. Indeed, serious autoimmune manifestations were seen in mice vaccinated with self-antigens expressed in the pancreatic islet cells or in the cardiomyocytes and arterial smooth muscle cells<sup>11</sup>. Cumulatively, these animal studies suggest that there is a difference in the susceptibility of normal and tumor tissue to the effector arms of the immune response. Therefore, vaccination against self-antigens expressed in tumor cells should be capable of inducing therapeutic immunity in the absence of devastating autoimmune consequences. The differences in susceptibility, however, are quantitative and, depending on the cellular target of the autoimmune response, increasing the effectiveness and/or intensity of vaccination with self-antigen could lead from mild to serious autoimmune pathology.

A judicious choice of antigens is key to alleviating concerns about destroying tolerance to self-tumor antigens. There are two groups of self-antigens that would make excellent choices for cancer vaccination, both of which should be effective and carry a minimal risk of autoimmunity. One group consists of self-antigens that are expressed in



immunoprivileged sites (such as the testis), which are reactivated in cancer cells. Several tumor antigens belonging to this category, such as the MAGE family of antigens, have been characterized in human cancers, foremost in melanoma. Another group consist of fetal or embryonic antigens that are re-expressed in cancer cells (such as carcinoembryonic antigen or oncofetal antigen). Antigens belonging to both groups are not expected to trigger tolerance nor induce autoimmunity.

The immune response to tumors is self-contained because tumors, even after partial decimation by a vaccination-induced immune response, are unable to channel antigens to the professional APC system that is needed to activate and reactivate the naïve and memory T cells sequestered in the secondary lymphoid organs<sup>32-34</sup>. Thus, tumor immunity and autoimmunity will not become a "runaway" response and it should be possible to control the development or severity of any autoimmune reactions simply by stopping vaccination (and therefore terminating the immune response).

In summary, tumor vaccination *via* the targeting of self-antigens expressed in tumor cells can be achieved without undue autoimmune pathology, largely due to an apparent difference between the requirements for inducing tumor regression and autoimmune pathology. Such treatments are, however, not without associated risks or the need for compromises and will require well thought-out choices to be made on a case-by-case basis.

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## APPENDIX B

(B1, B2, B3)



APPENDIX B1

# Tumor-specific killer cells in paraneoplastic cerebellar degeneration

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Models for immune-mediated tumor regression in mice have defined an essential role for cytotoxic T lymphocytes (CTLs); however, naturally occurring tumor immunity in humans is poorly understood<sup>1</sup>. Patients with paraneoplastic cerebellar degeneration (PCD) provide an opportunity to explore the mechanisms underlying tumor immunity to breast and ovarian cancer. Although tumor immunity and autoimmune neuronal degeneration in PCD correlates with a specific antibody response to the tumor and brain antigen cdr2<sup>2,3</sup>, this humoral response has not been shown to be pathogenic<sup>3,4</sup>. Here we present evidence for a specific cellular immune response in PCD patients. We have detected expanded populations of MHC class I-restricted cdr2-specific CTLs in the blood of 3/3 HLA-A2.1<sup>+</sup> PCD patients, providing the first description, to our knowledge, of tumor-specific CTLs using primary human cells in a simple recall assay. Cross-presentation of apoptotic cells by dendritic cells also led to a potent CTL response. These results indicate a model whereby immature dendritic cells that engulf apoptotic tumor cells can mature and migrate to draining lymph organs where they could induce a CTL response to tissue-restricted antigens. In PCD, peripheral activation of cdr2-specific CTLs is likely to contribute to the subsequent development of the autoimmune neuronal degeneration.

We examined the nature of the immune responses of three HLA-A2.1<sup>+</sup> PCD patients (patients 1–3) and one HLA-A2.1<sup>+</sup> PCD patient (patient 4). Patient 1 was seen during the acute phase of the disorder, and patients 2–4 had chronic disease at admission; they were seen 18 days, 9 months, 6 months and 5 months, respectively, after the onset of cerebellar dysfunction. After being diagnosed with PCD, all four patients were found to have gynecologic cancers: patient 1 had breast cancer and patients 2–4 had ovarian carcinoma. PCD is characterized by the presence of a high-titer antibody that is present in a patient's serum and spinal fluid; this antibody recognizes target antigens in Purkinje neurons and in gynecologic tumors (onconeural antigens; ref. 3). PCD antiserum has been used to clone cDNAs encoding immunoreactive antigens cdr1–3 (refs. 5–7), but PCD tumors express only the cdr2 antigen<sup>8</sup>. Thus, we confirmed the diagnosis of PCD in these patients by demonstrating the presence of high-titer cdr2 antibodies reactive with cloned cdr2 fusion protein (Fig. 1 and data not shown). For patient 1, a tumor block was available; by western blot analysis, the tumor was shown to express cdr2 (data not shown). Historically, all PCD tumors analyzed have been shown to express the cdr2 antigen<sup>8,9</sup>. Expression of cdr2 is normally tightly restricted to immune-privileged sites (neurons and testis) (ref. 8).

Initially, the discovery of onconeural antibodies led to the pro-

posal that PCD is an autoimmune disorder mediated by the humoral arm of the immune system. However, several observations indicate that cdr2 antibodies are not sufficient to cause disease. Treatments that reduce antibody titers are ineffective<sup>4</sup> and passive transfer of antibody does not reproduce the disorder in animals<sup>3</sup>. Furthermore, cdr2 is an intracellular protein, and it is unclear how such an antibody could mediate disruption of cellular function. To determine whether CD8<sup>+</sup> CTLs are involved in tumor immunity in PCD, we analyzed lymphocytes from the peripheral blood of patients. Peptide epitopes derived from cdr2 were 'pulsed' onto target cells, and antigen-specific cytotoxicity was measured in a standard <sup>51</sup>Cr-release assay. In patient 1, cdr2-specific CTLs were detected with specificity for the cdr2-2 peptide, and, to a lesser extent, the cdr2-1 peptide (Fig. 2a). This response was titratable and specific for acute PCD, as no response was detected in an HLA-A2.1<sup>+</sup> normal control (Fig. 2a) or in the patients with chronic PCD (data not shown).

To determine whether memory T cells were present in the peripheral blood of PCD patients, we established an *in vitro* recall assay. We prepared mature terminally differentiated dendritic cells<sup>10</sup> (DCs). The DCs generated had a typical stellate morphology, were nonadherent, expressed characteristic maturation markers (CD83), and had potent T cell-stimulating capacity in mixed leukocyte reactions at stimulator-to-responder ratios of 300:1 or less (data not shown). These blood-derived DCs were 'pulsed' with four different cdr2 peptides and co-cultured with purified syngeneic T cells. After 7 days, responding T cells were tested for cytolytic activity specific for cdr2 epitopes using 'peptide-pulsed' T2 (TAP<sup>+</sup>, HLA-A2.1<sup>+</sup>, class II<sup>+</sup>) cells as targets. In patients 2 and 3 (with chronic PCD), cdr2-specific CTLs were detected (Fig. 2b) using the cdr2-1 and cdr2-2 peptides. This CTL activity was not detected in the patient with acute PCD (patient 1), the HLA-A2.1<sup>+</sup> chronic PCD patient (patient 4), nor in five HLA-A2.1<sup>+</sup> control individuals tested (Fig. 2b and data not shown). As a positive control for these experiments, CTL responses specific for the immunodominant HLA-A2.1 epitope derived from the influenza matrix protein were determined (data not shown). Taken together, these data demonstrate the presence of an expanded population of class I-restricted cdr2-specific CTLs in the blood of both acute and chronically ill PCD patients.

To better understand how a tumor-specific antigen such as cdr2 might activate naive CTLs, we used an *in vitro* model for cross-presentation<sup>11</sup>. Apoptotic cells have been shown to serve as an essential trigger for the cross-presentation of epitopes derived from tissue-restricted antigens onto class I MHC molecules of professional antigen-presenting cells. HeLa cells, which express cdr2 (ref. 6), served as a source of antigen, and DCs were used as

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**Fig. 1** Western blot analysis of serum and CSF immunoreactivity to cdr2. Sera and CSF of patient 1 (lanes 1 and 4) and patient 2 (lanes 2 and 5) were tested for immunoreactivity to the cloned cdr2 fusion protein. As a specificity control, serum from a patient with an irrelevant PND (Hu syndrome) was also blotted (lane 3). Serum and CSF from patient 3 showed similar immunoreactivity (data not shown).

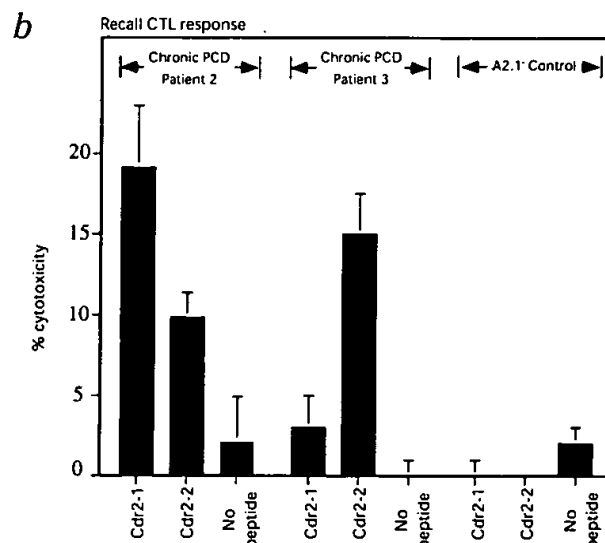
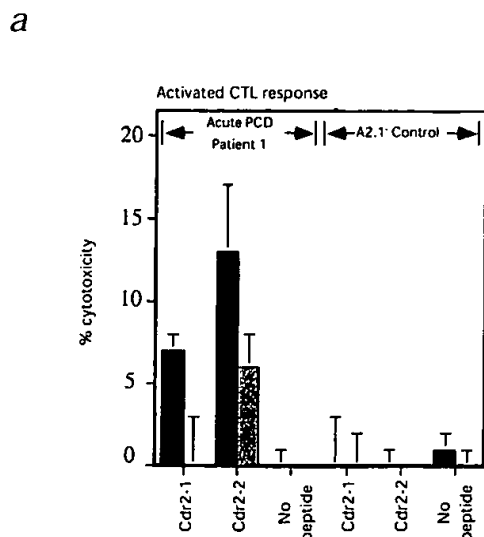


antigen-presenting cells for the induction of CTLs from the peripheral blood of patients with PCD. HeLa cells were induced to undergo apoptosis using ultraviolet B irradiation<sup>11</sup>. After 6–8 hours, DCs and T cells purified from patients 1 and 4 were added to the HeLa cell cultures. Responding T cells were collected after 7 days, and cytolytic activity specific for cdr2 epitopes was tested (Fig. 3). CTL activity specific for cdr2 epitopes was found in the HLA-A2.1<sup>+</sup> PCD patient (patient 1), but not in the HLA-A2.1<sup>+</sup> individual (patient 4), indicating that cell killing was MHC class I-restricted. CTLs were not generated to a control peptide (influenza matrix peptide, MP), indicating antigen specificity of the response. Reactivity was not detected against cdr2-2 peptide in this assay (data not shown), for reasons not yet understood. The percent killing demonstrated here is substantially higher than that detected using 'peptide-pulsed' DCs. We believe that the potent CTL response reflects increased efficiency by which antigens derived from apoptotic cells charge MHC-I on DCs relative to exogenous peptide (K. Inaba *et al.*, submitted). Additionally, the ability to stimulate CD4<sup>+</sup> T cells in these co-cultures may contribute to enhanced induction of cdr2-specific CTLs (ref. 12). Although a peptide derived from a HeLa antigen

other than cdr2 possibly served as the epitope for the activation of the cdr2-specific T cells, the use of 'unpulsed' and 'peptide-pulsed' T2 cells as targets in the <sup>51</sup>Cr-release assay conclusively demonstrates the expansion of HLA-A2.1-restricted cdr2-specific cells in patient 1.

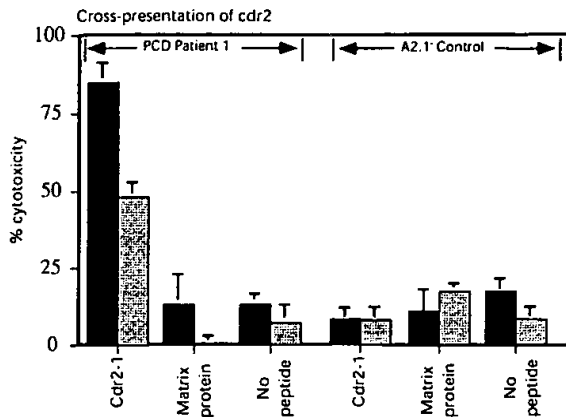
It is well-documented through animal models and clinical experience that the immune system can recognize and kill tumor cells<sup>1</sup>. CTLs are believed to have an essential role in this immune response, and the induction of tumor antigen-specific CTLs for immunotherapy constitutes an emerging strategy for treating cancer patients. However, expanded populations of tumor-specific CTLs have not previously been found in humans. In melanoma, CTL precursors specific for tumor antigens are not expanded in patients with cancer compared with those in normal individuals<sup>13</sup>. Indeed, established T-cell lines specific from defined tumor antigens could only be generated by repeated stimulation with antigen, reflecting *in vitro* priming rather than a true recall response<sup>1</sup>. This is in contrast to individuals who are infected with a virus such as HIV or influenza, in whom CTL precursor numbers are greatly expanded<sup>14–16</sup>. One reason for this discrepancy may be that the cancer patients studied have not mounted effective immune responses to their tumors; tumors such as melanoma contain restricted antigens for T cells, but they fail to trigger immunity.

Here we have examined the blood of PCD patients for evidence of cellular immunity, providing the first evidence, to our knowledge, for tumor-specific CTLs in patients with clinically evident tumor immunity. Paraneoplastic neurologic disorders (PNDs) are associated with tumor cell expression of neuron-specific proteins, and cDNAs encoding the respective onconeural antigens have been cloned<sup>3</sup>. PND patients typically come to clinical attention with severe neurologic dysfunction,



**Fig. 2** Peripheral blood of PCD patients contains cdr2-specific killer cells. T cells were isolated from the peripheral blood of acute (a) and chronic (b) HLA-A2.1<sup>+</sup> PCD patients. a, These purified T cells were used directly in a <sup>51</sup>Cr-release assay using 'peptide-pulsed' T2 cells as targets. Peptides cdr2-1 and cdr2-2 were predicted based on known anchor residues for the HLA-A2.1 binding groove. Effector:Target ratios are 100:1 (filled bars) and 30:1 (shaded bar). b, Blood-derived dendritic cells were generated from PCD and HLA-A2.1<sup>+</sup> matched control individuals. These DCs were 'pulsed' with the cdr2-1 and cdr2-2 peptides and co-cultured

with T cells. After 7 days, the responding T cells were tested for cytolytic activity specific for cdr2 as determined in a standard <sup>51</sup>Cr-release assay. The HLA-A2.1 immunodominant epitope derived from the influenza matrix protein served as a positive control for the generation of a CTL recall response (data not shown). Effector:Target ratio is 20:1. In a and b, percent cytotoxicity is measured as a function of spontaneous and total release. Background killing of target cells was 0–2% in all groups. Results are representative of four experiments, and each value represents the mean from triplicate wells.



**Fig. 3** Enhanced CTL activity is detected when apoptotic HeLa cells are the source of cdr2 epitopes. HeLa cells (filled bar),  $1 \times 10^4$ ; shaded bar,  $1 \times 10^5$  were ultraviolet B-irradiated to induce apoptosis, and DCs and T cells purified from a PCD patient were then added the apoptotic HeLa cells. Responding T cells were tested in a cytotoxicity assay. Percent killing of 'peptide-pulsed' T2 cells is shown using both no peptide and MP as negative controls. Patient 4, who is HLA-A2.1<sup>+</sup>, had no detectable cdr2-specific response. Data are representative of two experiments, and each value represents the mean from triplicate wells.

unaware that they have a tumor, and in some cases PND-associated tumors have been documented to regress with the onset of autoimmune neurologic disease<sup>17</sup>. Among patients with PCD, two-thirds present with neurologic symptoms before the diagnosis of cancer, and nearly 90% have limited oncologic disease when diagnosed; by comparison, only 50–60% of unselected breast cancer patients and 25% of ovarian cancer patients present with limited stage disease<sup>18</sup>.

In three of three HLA-A2.1<sup>+</sup> PCD patients, we have found cytotoxic T cells that specifically lyse target cells presenting HLA-A2.1<sup>+</sup>-restricted peptides derived from the PCD-cdr2 antigen. Given that tumor cells themselves are unable to activate naive CTLs (ref. 19), professional antigen-presenting cells are likely to be essential for the induction of cdr2-specific CTLs in PCD (ref. 20). Based on our *in vitro* data (Fig. 3) and the *in vivo* models of others<sup>19</sup>, cross-presentation of tumor antigens could account for the initial stimulation of the CTLs in PCD. DCs can acquire apoptotic cells and effectively cross-present intracellular viral antigens derived from the apoptotic material on MHC I, stimulating class I-restricted CTLs (ref. 11). We have extended these observations by using cdr2-expressing apoptotic tumor cells as a source of antigenic material.

These observations indicate a mechanism whereby tumor antigen-specific T cells may be generated after expression of restricted antigens. Apoptotic tumor cells phagocytosed by peripheral tissue dendritic cells may cross-present epitopes derived from such tumor antigens on class I MHC molecules. After migrating to a draining lymph node, such DCs might engage antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells. After being activated, such T cells may return to the tumor site and lyse tumor cells. In PCD, cdr2 may be an effective antigen for the induction of tumor immunity, due in part to the absolute restriction of its expression to immune-privileged sites throughout development<sup>8</sup>. Additional factors are also necessary for the immune response, including tumor cell expression of MHC-I (found in a high percentage of PND-associated tumors<sup>21</sup>) and lack of tumor-cell ex-

pression of proteins capable of inducing T-cell tolerance, such as Fas ligand<sup>22</sup>.

The detection of cdr2-specific CTLs in PCD patients indicates a new model for PCD pathogenesis. We suggest that cdr2-specific T cells are induced by the tumor cell expression of a protein normally expressed in immune-privileged tissues, leading to the observed tumor immunity. Such cells are likely to contribute to neuronal degeneration in PCD; this is supported by reports of MHC I expression in neurons<sup>23</sup>, including Purkinje neurons, and by detection of CTLs in the CSF of PCD patients early in the course of disease (M.L.A. and R.B.D., unpublished data).

## Methods

**Monoclonal antibodies.** Monoclonal antibodies to these antigens were used: CD8, CD14, HLA-DR (Becton Dickinson, San Jose, California) and CD83 (Coulter, Hialeah, Florida).

**Clinical samples.** All patients were self-referred after a diagnosis of PCD, and were seen at The Rockefeller University Hospital General Clinical Research Center. After informed consent was obtained, blood was drawn for analysis. Patient 1 was seen 18 days after the onset of cerebellar symptoms. At that time, she was able to speak with considerable dysarthria, could use her hands to pick up large objects, but otherwise she was completely bedridden with pancerebellar signs and symptoms. Patients 2–4 had chronic cerebellar disease and were clinically typical of individuals with PCD.

**Generation of mononuclear cell subsets.** Blood was collected in heparinized syringes or by leukapheresis. Peripheral blood mononuclear cells were isolated using Ficoll-Hypaque (Pharmacia). T cell-enriched and T cell-depleted populations were prepared by 'rosetting' with neuraminidase-treated sheep red blood cells as described<sup>24</sup>. T cells were further purified from T cell-enriched cells for the CTL recall assays by removing monocytes, natural killer (NK) cells, and B cells as described<sup>24</sup>. DCs were generated from peripheral blood precursors by culturing T cell-depleted cells for 7 days in the presence of GM-CSF (Immunex, Seattle, Washington) and IL-4 (Schering-Plough, Kenilworth, New Jersey), followed by culturing for 4 days in monocyte conditioned medium<sup>10</sup>.

**Synthetic peptides.** Human PCD peptides were predicted based on anchor residues for HLA A2.1 (ref. 25) and were synthesized for use in cytotoxicity assays (Biosynthesis, Lewisville, Texas). These peptides are (numbering is relative to the full-length mouse cdr2 sequence<sup>8</sup>; GeneBank accession # 1857921): cdr2-1 (KLVPDSLYV; amino acids 273–281), cdr2-2 (SLLEEMFLT; amino acids 289–297), cdr2-3 (QMLQSEHPFV; amino acids 259–268) and cdr2-4 (SLLEEMFLTV; amino acids 289–298). The HLA-A2.1-restricted immunodominant peptide derived from the influenza matrix protein MP (GILGFVFTL) served as a control<sup>11</sup>.

**Cytotoxicity assays.** Activated CTLs were detected using T cells as effector cells in a conventional  $\text{Na}^{51}\text{CrO}_4$ -release assay directly after purification. T2 cells (a TAP<sup>+</sup>, HLA-A2.1<sup>+</sup>, class II<sup>+</sup> cell line) were 'pulsed' for 1 hour with 1  $\mu\text{M}$  of various peptides, loaded with  $\text{Na}^{51}\text{CrO}_4$ , and used as targets<sup>24</sup>. Alternatively, memory CTL responses were stimulated using DCs 'pulsed' for 2–4 hours with 1  $\mu\text{M}$  of various peptides at 25 °C. 'Peptide-pulsed' DCs were then cultured with purified T cells, and after 7 days, responding T cells were assayed for cytolytic activity. Again, T2 cells 'pulsed' with peptide served as targets<sup>24</sup>. In cross-presentation cytotoxicity experiments, HeLa cells were triggered to undergo apoptosis using an ultraviolet b lamp (model# 60UVB; Derma Control, Dolton, Illinois), calibrated to provide 2  $\text{mJ}/\text{cm}^2$  per second (ref. 11). These cells served as a source of cdr2 antigen and were co-cultured with DCs and T cells prepared from PCD patients. After 7 days, responding T cells were assayed for cytolytic activity using 'peptide-pulsed' T2 cells as targets. In all CTL assays, percent cytotoxicity was determined by using the average values of triplicates from experimental wells (E) as compared to average values of spontaneous (S) and total (T) release as follows: % cytotoxicity =  $[(E-S)/(T-S)] \times 100$ . Background lysis is shown in all experiments and ranged from 0% to 3% in the activated and

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memory CTLs, and from 5% to 22% in the cross-presentation assays, presumably because of a cross-over reaction between HeLa derived and T2 generated peptides.

**Western blot analysis.** The human cdr2 fusion protein produced from a full-length cDNA (ref. 6) was run on a 10% SDS-PAGE, transferred to nitrocellulose, and blotted against the serum (1:10,000 dilution) and CSF (1:500 dilution) of PCD patients. As a specificity control, serum (1:500 dilution) from a patient with an irrelevant PND (Hu syndrome) was also blotted.

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## REVIEW ARTICLE

## MECHANISMS OF DISEASE

## Paraneoplastic Syndromes Involving the Nervous System

Robert B. Darnell, M.D., Ph.D., and Jerome B. Posner, M.D.

THE TERM "PARANEOPLASTIC SYNDROMES" REFERS TO SYMPTOMS OR signs resulting from damage to organs or tissues that are remote from the site of a malignant neoplasm or its metastases. Paraneoplastic syndromes can affect most organs and tissues. Widely known examples include cancer cachexia,<sup>1</sup> hypercalcemia,<sup>2</sup> Cushing's syndrome,<sup>3</sup> and Trousseau's syndrome.<sup>4</sup> Most of these paraneoplastic syndromes occur because the tumor secretes substances that mimic normal hormones or that interfere with circulating proteins. A few paraneoplastic neurologic disorders are caused by similar mechanisms (e.g., carcinoid myopathy and encephalopathy).<sup>5</sup> However, most or all paraneoplastic neurologic disorders are immune-mediated. (We do not consider damage to the nervous system by cancer-induced coagulopathies or opportunistic infections to be paraneoplastic neurologic disorders.) The cancers causing paraneoplastic neurologic disorders are often asymptomatic and sometimes occult; it is the neurologic symptoms that take the patient to the doctor. The combination of an indolent tumor and severe neurologic disability suggests effective antitumor immunity coupled with autoimmune brain degeneration. This review describes paraneoplastic neurologic disorders believed to be immune-mediated and discusses our current understanding of their mechanisms.

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## CLINICAL FEATURES

Paraneoplastic neurologic disorders can affect any part of the nervous system (Table 1). Some of them affect only a single area (e.g., limbic encephalitis) or a single cell type (e.g., the Purkinje cells of the cerebellum). In other instances, multiple levels of the nervous system are involved (e.g., encephalomyeloradiculitis).

Most symptomatic paraneoplastic syndromes are rare, affecting perhaps 0.01 percent of patients with cancer. Exceptions are the Lambert-Eaton myasthenic syndrome, which affects about 3 percent of patients with small-cell lung cancer<sup>35</sup>; myasthenia gravis, which affects about 15 percent of patients with thymoma<sup>36</sup>; and demyelinating peripheral neuropathy, which affects about 50 percent of patients with the rare osteosclerotic form of plasmacytoma (the polyneuropathy, organomegaly, endocrinopathy, M protein, and skin changes [POEMS] syndrome).<sup>37</sup> Clinical and electrophysiological studies in patients with cancer, particularly small-cell lung cancer, often disclose proximal muscle weakness or delayed conduction along peripheral nerves in asymptomatic patients.<sup>38</sup> Whether these abnormalities are true paraneoplastic neurologic disorders is unknown.

The symptoms and signs of paraneoplastic syndromes are diverse, but certain features are common. The neurologic disorder usually appears before the cancer has been identified. In many instances an initial search for cancer is unrewarding; the tumor is found months or even a few years after the appearance of the neurologic syndrome. Whole-body positron-emission tomography may be the best screening method for locat-

Table 1. Paraneoplastic Syndromes of the Nervous System.

Location of Syndrome	Reference
<b>Brain and cranial nerves</b>	
Limbic encephalitis	Gultekin et al. <sup>6</sup>
Brain-stem encephalitis	Barnett et al. <sup>7</sup>
Cerebellar degeneration	Peterson et al., <sup>8</sup> Cao et al. <sup>9</sup>
Opsoclonus–myoclonus	Bataller et al. <sup>10</sup>
<b>Visual syndromes</b>	
Cancer-associated retinopathy	Goldstein et al. <sup>11</sup>
Optic neuritis	Lieberman et al. <sup>12</sup>
Chorea	Croteau et al. <sup>13</sup>
Parkinsonism	Golbe et al. <sup>14</sup>
<b>Spinal cord</b>	
Necrotizing myelopathy	Rudnicki and Dalmau <sup>15</sup>
Inflammatory myelitis	Babikian et al., <sup>16</sup> Hedges et al. <sup>17</sup>
Motor neuron disease (amyotrophic lateral sclerosis)	Younger <sup>18</sup>
Subacute motor neuropathy	Schold et al. <sup>19</sup>
Stiff-person syndrome	Brown and Marsden, <sup>20</sup> Silverman <sup>21</sup>
<b>Dorsal-root ganglia</b>	
Sensory neuropathy	Graus et al. <sup>22</sup>
<b>Peripheral nerves</b>	Rudnicki and Dalmau, <sup>15</sup> Antoine et al. <sup>23</sup>
Autonomic neuropathy	Lee et al. <sup>24</sup>
Acute sensorimotor neuropathy	
Polyradiculoneuropathy (Guillain-Barré syndrome)	Lisak et al. <sup>25</sup>
Brachial neuritis	Lachance et al. <sup>26</sup>
Chronic sensorimotor neuropathy	Antoine et al. <sup>23</sup>
Vasculitic neuropathy	Blumenthal et al. <sup>27</sup>
Neuromyotonia	Lahrman et al., <sup>28</sup> Vincent <sup>29</sup>
<b>Neuromuscular junction</b>	
Lambert–Eaton myasthenic syndrome	Carpentier and Delattre <sup>30</sup>
Myasthenia gravis	Vernino et al. <sup>31</sup>
<b>Muscle</b>	
Polymyositis or dermatomyositis	Stockton et al. <sup>32</sup>
Necrotizing myopathy	Levin et al. <sup>33</sup>
Myotonia	Pascual et al. <sup>34</sup>

ing the occult cancer.<sup>39</sup> Although the tumor may be indolent,<sup>40</sup> the neurologic illness usually develops rapidly over days to a few months. Paraneoplastic neurologic disorders are usually severe, often disabling, and sometimes lethal.<sup>22</sup>

#### LABORATORY FINDINGS

Examination of cerebrospinal fluid reveals a mild pleocytosis (30 to 40 white cells per cubic milli-

meter), a slightly elevated protein level (50 to 100 mg per deciliter), and an elevated IgG level. Pleocytosis is usually apparent only early in the course of the disease and disappears within several weeks to months. The elevated IgG level may, however, persist. Analysis of cerebrospinal fluid cells in patients with paraneoplastic cerebellar degeneration through fluorescent-activated cell sorting has revealed that the predominant cell type (over 75 percent) is T cells, with a small component (less than 10 percent) of B cells and natural killer cells.<sup>41</sup>

#### ANTIBODIES

Perhaps most important diagnostically, many patients with paraneoplastic syndromes have antibodies in their serum (and cerebrospinal fluid) that react with both the nervous system and the underlying cancer (Fig. 1 and Table 2). The identification of these antibodies and their target neural antigens has substantially advanced our ability to make an early diagnosis and has led to the concept that paraneoplastic neurologic disorders are immune-mediated.

Although there is considerable overlap, each of these antibodies is associated with a narrow spectrum of clinical syndromes and a restricted subgroup of cancers (Table 2). The antibodies, some of which we named using the first two letters of the surname of the index patient, are highly specific for identifying a patient with neurologic disability who has a paraneoplastic syndrome. These antibodies also suggest the site of the underlying cancer. For example, the presence of anti-Yo antibodies in the serum of a woman with cerebellar symptoms is virtually conclusive evidence that she has paraneoplastic cerebellar degeneration and gynecologic, usually ovarian, cancer (Fig. 1A).

Unfortunately, not all patients with paraneoplastic syndromes have identifiable antibodies in their serum. Whether this is a technical fault in detection or whether some paraneoplastic neurologic disorders are not immune-mediated is not known.

#### ANTIGENS

In most cases of paraneoplastic syndromes associated with antibodies, the antigen has been identified and the gene coding for the antigen has been cloned and sequenced (Table 2). Some of these antigens are expressed by all tumors of a given histologic type, whether or not the patient mounts an immune response against them. Other tumors rarely express such antigens unless the cancer causes a paraneoplastic neurologic disorder. Failure to find the anti-



gen in the cancer of a patient with paraneoplastic antibodies should prompt a search for a second cancer.<sup>22</sup>

#### PATHOPHYSIOLOGICAL FEATURES

##### THE AUTOIMMUNE MODEL OF PATHOGENESIS

Currently, it is thought that most or all paraneoplastic neurologic disorders are immune-mediated (Fig. 2). The mechanism entails ectopic expression by a tumor of an antigen that normally is expressed exclusively in the nervous system. Some of these so-called onconeural antigens are also expressed in the normal testis, an organ that is, like the brain, an immunologically privileged site. The tumor antigen is identical to the neural antigen,<sup>68</sup> but for unknown reasons the immune system identifies it as foreign and mounts an immune attack. The immune attack controls the growth of the cancer and may in a few instances obliterate it (Fig. 3). However, the antibodies and cytotoxic T cells that are specific for the tumor antigen are not sufficient to cause the neurologic disease unless they cross the blood-brain barrier and react with neurons expressing the onconeural antigen (Table 3).

##### TUMOR IMMUNITY IN PARANEOPLASTIC SYNDROMES

###### *The Tumor*

Onconeural antigens are present in the tumor in all patients with antibody-positive paraneoplastic neurologic disorders and in many patients without such disorders. Moreover, the genes for these antigens are not mutated in tumor cells.<sup>68,70,71</sup> Thus, paraneoplastic neurologic syndromes cannot be attributed to the infrequency of expression of the relevant tumor antigens or to mutations in the genes encoding these antigens.

The tumor is often occult, and the neurologic disorder typically precedes the diagnosis of the tumor.<sup>8,22</sup> For example, patients with the Hu paraneoplastic syndrome typically harbor small-cell lung cancers that are limited to single nodules (53 of 55 patients in one study<sup>44</sup>), despite the fact that most small-cell lung cancers (over 60 percent) are widely metastatic at diagnosis. In a few instances, unequivocal paraneoplastic syndromes may follow identification and even treatment of the tumor, and may sometimes herald a relapse.

The histologic features of tumors in paraneoplastic neurologic disorders do not differ from those of other tumors, except that the tumors may be

heavily infiltrated with inflammatory cells.<sup>8,72,73</sup> Many reports suggest that patients with paraneoplastic neurologic disorders have a better prognosis than patients with histologically identical tumors that are not associated with paraneoplastic neurologic disorders.<sup>74-77</sup> The improved prognosis is not simply a result of earlier diagnosis of the cancer because the neurologic disease has led to a search for cancer. Patients with low titers of anti-Hu antibodies but without paraneoplastic disorders also have more limited small-cell lung cancer than patients who do not have the antibodies.<sup>40,78</sup>

###### *The Nervous System*

The presence of antigen-specific cytotoxic T cells in paraneoplastic neurologic disorders was clearly documented after a patient with acute paraneoplastic cerebellar degeneration and anti-Yo antibodies was found to have activated T cells in her blood that were able to lyse target cells presenting the Yo (also called cdr2) antigen in vitro.<sup>79</sup> Subsequent studies in chronically ill patients with paraneoplastic cerebellar degeneration have used autologous antigen-presenting cells (dendritic cells) to reactivate responses to the cdr2 antigen in memory cytotoxic T cells. Such reactivated responses have been elicited in all patients with paraneoplastic cerebellar degeneration whose T cells were tested for the phenomenon.<sup>41,79</sup> These studies have been complemented by reports of a limited V $\beta$  chain T-cell repertoire in patients with the Hu syndrome (the V $\beta$  is one of the two chains, V $\beta$  and V $\alpha$ , of the T-cell receptor).<sup>80</sup> Taken together, the evidence indicates that T-cell responses have an important role in paraneoplastic neurologic disorders.

Antibodies in paraneoplastic neurologic disorders react with the portion of the nervous system that is responsible for the clinical symptoms — for example, anti-Purkinje-cell antibodies occur in patients with paraneoplastic cerebellar degeneration.<sup>81</sup> In many instances, the reaction is more widespread than the clinical findings. In paraneoplastic neurologic disorders affecting the brain, relatively high titers of the antibody in the cerebrospinal fluid (relative to total IgG) indicate that the antibody is synthesized within the brain, presumably by specific B cells that have crossed the blood-brain barrier.<sup>82</sup>

One report described the presence of anti-Hu antibodies within neuronal nuclei of the central nervous system in patients who died of their paraneoplastic syndromes.<sup>83</sup> Although some believe this

finding to be an artifact, antibodies to double-stranded DNA, the hallmark of systemic lupus erythematosus, have been found within the nuclei of cells in patients with systemic lupus erythematosus.<sup>84</sup>

#### *Antibodies and Cytotoxic T Cells*

The relative roles of humorally mediated immunity (antibodies) and cellular immunity (T cells) in paraneoplastic neurologic disorders are unresolved.<sup>85</sup> This uncertainty is complicated by the fact that different paraneoplastic neurologic disorders may have different underlying mechanisms. When the target antigens are cell-surface receptors, as in the Lambert-Eaton myasthenic syndrome, myasthenia gravis, and a rare form of paraneoplastic cerebellar degeneration, antibodies appear to have the predominant role.

### UNRESOLVED ISSUES

#### **ANIMAL MODELS**

Studies in animals have failed to reproduce paraneoplastic neurologic syndromes, perhaps in part because many of them have focused on antineuronal antibodies, whereas studies in humans have implicated an important cellular component in the immune response in several paraneoplastic neurologic syndromes. In one report, animals immunized with DNA corresponding to the Hu antigen were protected against subsequent inoculation of the tumor,<sup>86</sup> but the importance of this report, in the face of many similar reports in which protection was induced in animal models of tumors not associated with paraneoplastic neurologic disorders, is uncertain.

#### **PROTECTION AGAINST THE TUMOR**

It is not known whether the antitumor immune response in paraneoplastic neurologic disorders can be harnessed to treat tumors without damaging the nervous system. In the current model of paraneoplastic neurologic disorders (Fig. 2),<sup>87</sup> apoptosis of tumor cells triggers an antitumor immune response. Indeed, it has been shown that apoptotic tumor cells in paraneoplastic neurologic disorders are a potent means of activating tumor-specific T cells.<sup>88</sup> Such killer T cells could trigger a feedback loop by inducing apoptosis and hence amplification of the antitumor immune response. These observations suggest that understanding the mechanisms that trigger effective tumor immune responses in pa-

#### **Figure 1 (facing page). Two Different Antibodies in Paraneoplastic Syndromes.**

Panel A shows a magnetic resonance imaging (MRI) scan from a woman with an acute onset of cerebellar dysfunction (upper left-hand side). There is enhancement of cerebellar folia, suggesting an acute inflammatory reaction. Antibodies in her serum reacted with Purkinje cells of the cerebellum (brown staining, upper right-hand side; hematoxylin counterstain,  $\times 100$ ). Subsequently, an ovarian cancer was discovered. Antibodies in her serum reacted with the cancer cells (lower left-hand side; hematoxylin counterstain,  $\times 100$ ). Western blotting (lower right-hand side) against cerebellar Purkinje cells and the tumor revealed bands at 62 and 34 kD. Control serum did not react with Purkinje cells. Panel B shows an MRI scan from a woman with severe sensory neuropathy and loss of memory (upper left-hand side). It reveals hyperintensity in the medial temporal lobes on the T<sub>2</sub>-weighted image (left) and slight contrast enhancement in the right temporal lobe (arrow). Two mediastinal biopsies revealed only inflammation. Antibodies in her serum reacted with all neurons in the central and peripheral nervous system, staining the nucleus more strongly than the cytoplasm and sparing the nucleolus (upper right-hand side; hematoxylin counterstain,  $\times 200$ ). The woman died suddenly of acute autonomic failure. Autopsy showed a small-cell lung cancer that also reacted with antibodies in her serum (lower left-hand side; hematoxylin counterstain,  $\times 100$ ). Western blot analysis (lower right-hand side) showed that antibodies in her serum reacted with extracts of cortical neurons and of small-cell lung-cancer cells. Normal serum does not produce such a reaction.

tients with paraneoplastic neurologic disorders may have an important role in developing successful approaches to tumor immunotherapy.

#### **VARIATIONS IN PATHOLOGICAL FEATURES**

Another factor complicating our understanding of the neuronal degeneration in paraneoplastic neurologic disorders is the fact that the pathological features of these disorders vary widely. For example, in paraneoplastic cerebellar degeneration, there is total loss of the Purkinje cells of the cerebellum, with little or no pathological change elsewhere in the nervous system and no identifiable inflammatory infiltrates within the cerebellum itself. By contrast, in paraneoplastic encephalomyelitis, there is not only widespread destruction of neurons, including Purkinje cells, but also florid inflammation within the central nervous system and intraneuronal deposits of antibodies.<sup>83</sup> In some patients with paraneoplastic syndromes, particularly opsoclonus-myoclonus, autopsy may demonstrate an entirely normal brain

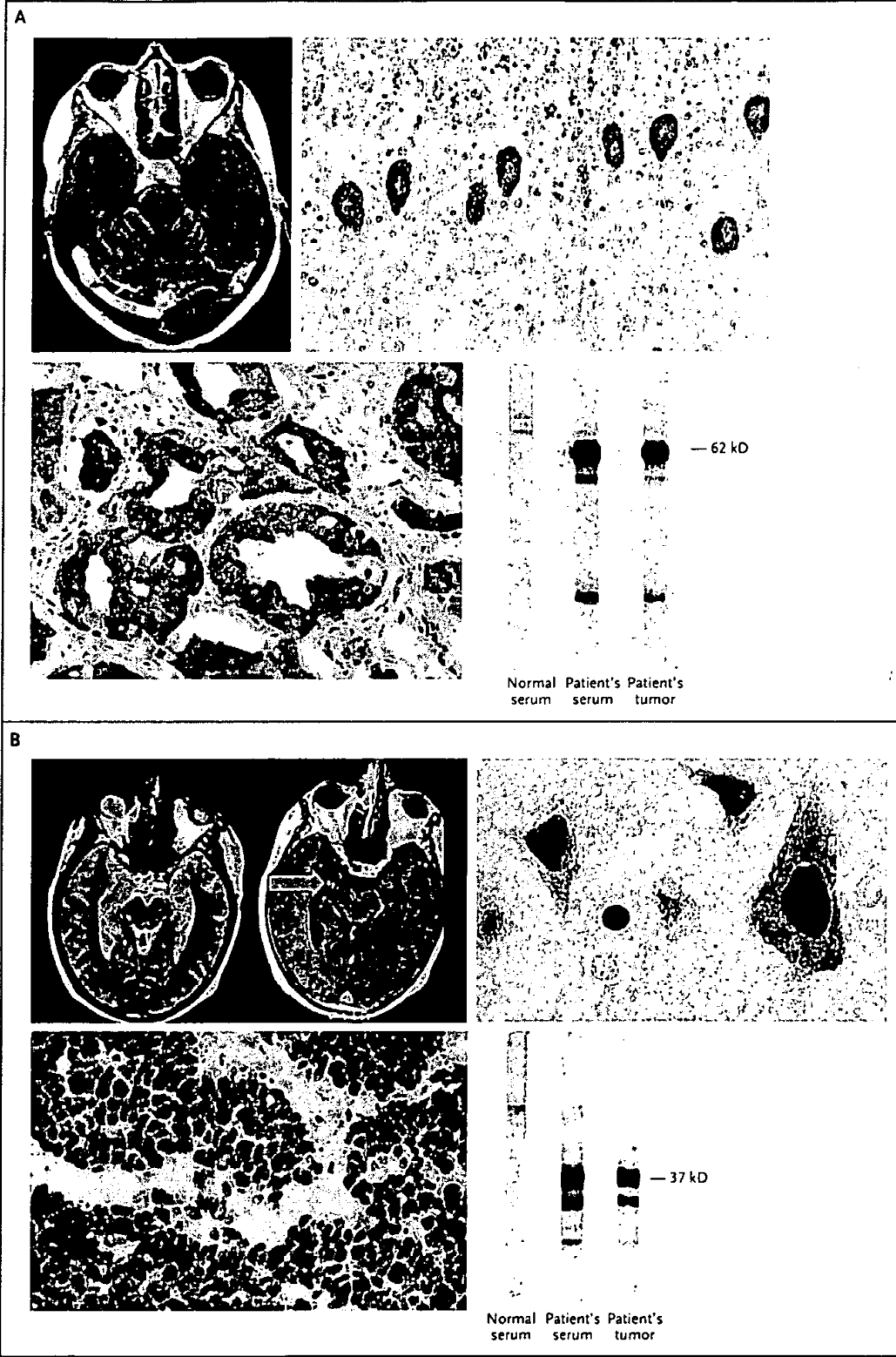


Table 2. Antineuronal-Antibody-Associated Paraneoplastic Disorders.\*

Antibody	Neuronal Reactivity	Protein Antigens	Cloned Genes	Tumor	Paraneoplastic Symptoms	References
Anti-Hu (ANNA-1)	Nucleus more than cytoplasm (all neurons)	35–40 kD	HuD, HuC, HuI-1	Small-cell lung cancer, neuroblastoma, prostate cancer	Paraneoplastic encephalomyelitis, paraneoplastic sensory neuronopathy, paraneoplastic cerebellar degeneration, autonomic dysfunction	Graus et al., <sup>22</sup> Dalmau et al., <sup>44</sup> Szabo et al., <sup>45</sup> Levine et al., <sup>46</sup> Sakai et al. <sup>47</sup>
Anti-Yo (PCA-1)	Cytoplasm, Purkinje cells	34 and 62 kD	CDR34, CDR62	Ovarian, breast, and lung cancers	Paraneoplastic cerebellar degeneration	Peterson et al., <sup>8</sup> Fathallah-Shaykh et al., <sup>48</sup> Darnell et al. <sup>49</sup>
Anti-Ri	Nucleus more than cytoplasm (central nervous system neurons)	55 and 80 kD	Nova	Breast, gynecologic, lung, and bladder cancers	Ataxia with or without opsoclonus-myoclonus	Jensen et al., <sup>50</sup> Yang et al., <sup>51</sup> Luque et al., <sup>52</sup> Buckanovich et al. <sup>53</sup>
Anti-Tr	Cytoplasm, Purkinje cells	?	—	Hodgkin's lymphoma	Paraneoplastic cerebellar degeneration	Peltola et al. <sup>54</sup>
Anti-VGCC	Presynaptic neuromuscular junction	64 kD	P/Q type VGCC, <i>MysB</i>	Small-cell lung cancer	Lambert-Eaton myasthenic syndrome	Carpentier and Delattre <sup>30</sup>
Antiretinal	Photoreceptors, ganglion cells	23, 65, 145, and 205 kD	Recoverin	Small-cell lung cancer, melanoma, gynecologic cancers	Cancer-associated retinopathy, melanoma-associated retinopathy	Maeda et al., <sup>55</sup> Polans et al., <sup>56</sup> Thirkill et al. <sup>57</sup>
Anti-amphiphysin	Presynaptic nerve terminals	128 kD	Amphiphysin	Breast cancer, small-cell lung cancer	Stiff-person syndrome, paraneoplastic encephalomyelitis	Saiz et al., <sup>58</sup> De Camilli et al., <sup>59</sup> Folli et al. <sup>60</sup>
Anti-CRMP5 (Anti-CV2)	Oligodendrocytes, neurons, cytoplasm	66 kD	CRMP5 (POP66)	Small-cell lung cancer, thymoma	Encephalomyelitis, cerebellar degeneration, chorea, sensory neuropathy	Yu et al. <sup>61</sup>
Anti-PCA-2	Purkinje cytoplasm and other neurons	280 kD	—	Small-cell lung cancer	Encephalomyelitis, cerebellar degeneration, Lambert-Eaton myasthenic syndrome	Battaller et al. <sup>10</sup>
Anti-Ma1	Neurons (subnucleus)	40 kD	Ma1	Lung cancer, other cancers	Brain-stem encephalitis, cerebellar degeneration	Rosenfeld et al. <sup>62</sup>
Anti-Ma2	Neurons (subnucleus)	41.5 kD	Ma2	Testicular cancer	Limbic brain-stem encephalitis	Rosenfeld et al. <sup>62</sup>
ANNA-3	Nuclei, Purkinje cells	170 kD	—	Lung cancer	Sensory neuronopathy, encephalomyelitis	Chan et al. <sup>63</sup>
Anti-mGluR1	Purkinje cells, olfactory neurons, hippocampus	Metabotropic glutamate receptor	Glu receptor	Hodgkin's lymphoma	Paraneoplastic cerebellar degeneration	Smitt et al. <sup>64</sup>
Anti-VGCC	Peripheral nerve	VGCC	Potassium channels	Thymoma, small-cell lung cancer	Neuromyotonia	Vernino and Lennon, <sup>65</sup> Hart et al. <sup>66</sup>
Anti-MAG	Peripheral nerve	MAG	MAG	Waldenström's macroglobulinemia	Peripheral neuropathy	Vitala <sup>67</sup>

\* There is no uniform nomenclature for some of the antibodies.<sup>42,43</sup> In this article, we use the nomenclature developed in our laboratory. Where differences exist, they are indicated in parentheses. VGCC denotes voltage-gated calcium channel, VGKC voltage-gated potassium channel, and MAG myelin-associated glycoprotein.

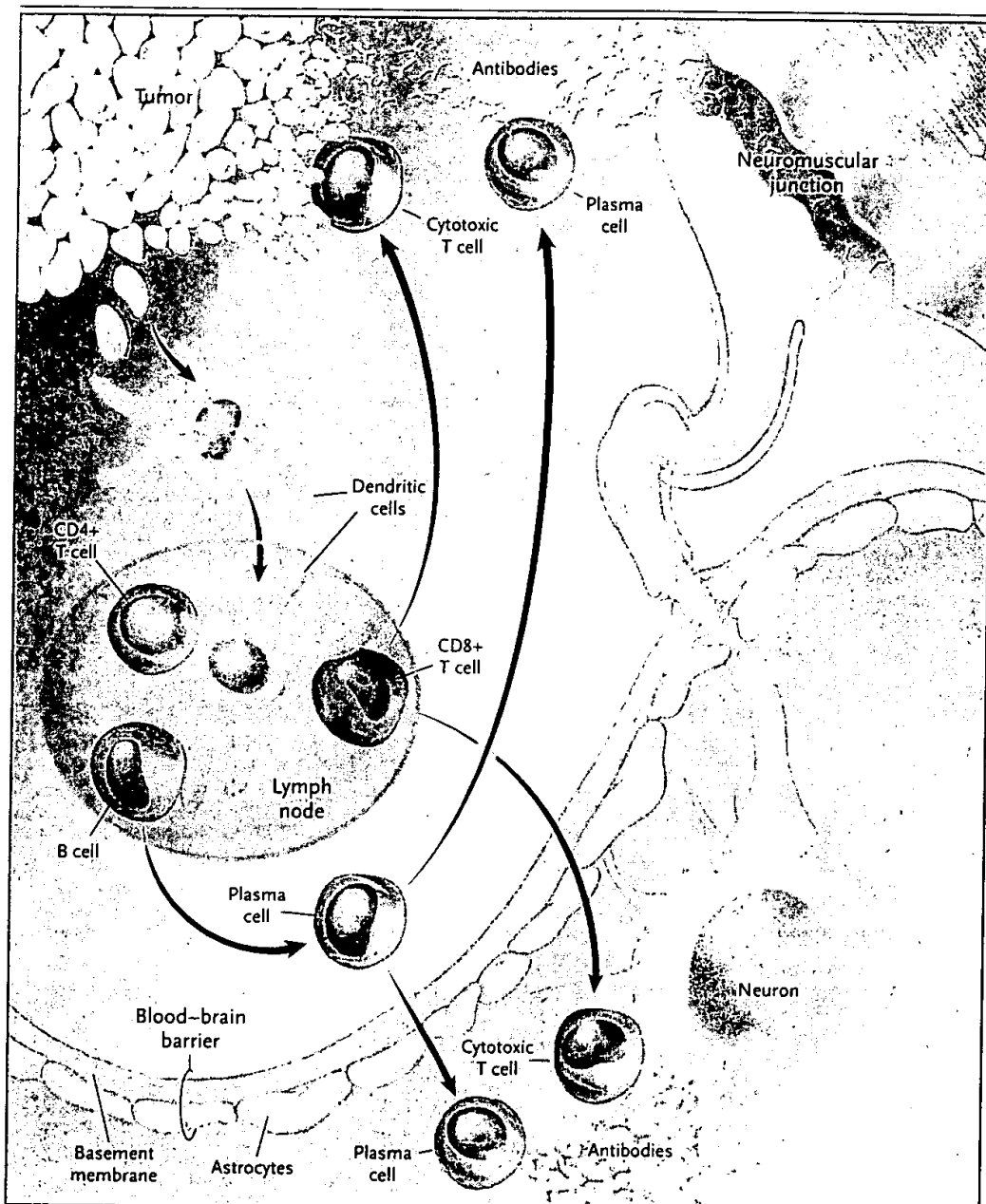
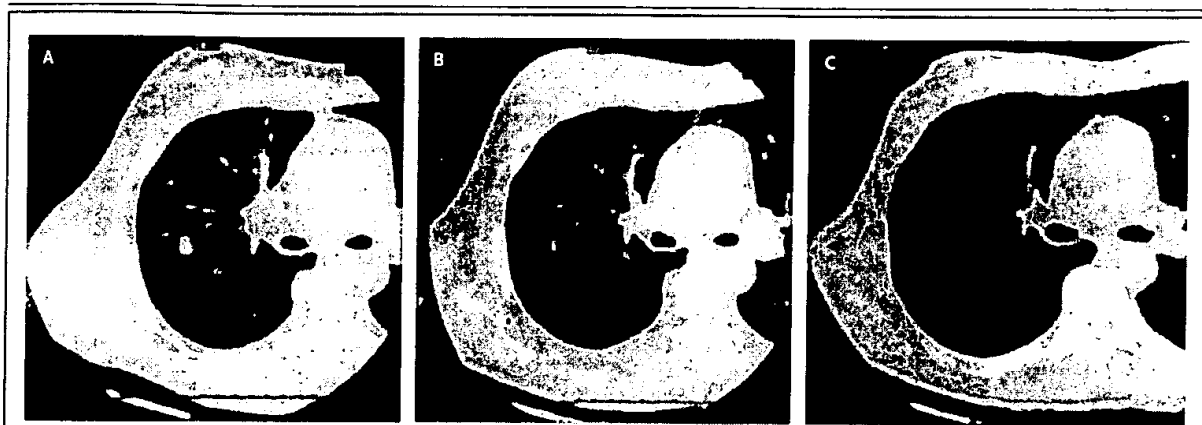


Figure 2. Proposed Pathogenesis of Paraneoplastic Neurologic Disorders.

A tumor not involving the nervous system expresses a neuronal protein that the immune system recognizes as nonself. Apoptotic tumor cells are phagocytized by dendritic cells that migrate to lymph nodes, where they activate antigen-specific CD4+, CD8+, and B cells. The B cells mature into plasma cells that produce antibodies against the tumor antigen. The antibodies or the cytotoxic CD8+ T cells (or both) slow the growth of the tumor, but they also react with portions of the nervous system outside the blood-brain barrier. In the illustration, antibodies are reacting with voltage-gated calcium channels at the neuromuscular junction, causing the Lambert-Eaton myasthenic syndrome. In some instances, plasma cells and cytotoxic T cells cross the blood-brain barrier and attack neurons expressing the antigen they share with the tumor.



**Figure 3. Spontaneous Regression of Lung Lesions in a Patient with Encephalomyelitis and Anti-Hu Antibodies.**

The computed tomographic (CT) scan in Panel A shows a lung mass with hilar and mediastinal adenopathy. During the course of the workup, sensory loss and cerebellar signs developed and anti-Hu antibodies were found in the serum. A second CT scan, obtained before any treatment was administered (Panel B), shows partial resolution of the lung lesion and the adenopathy. A right-upper-lobe lobectomy yielded only fibrous tissue and inflammation. The adenopathy then resolved without further treatment (Panel C). The patient's clinical symptoms also began to improve, and she was left with only mild cerebellar signs. This case is described as Case 1 in Byrne et al.<sup>69</sup> Scans courtesy of Dr. Thomas Byrne.

even when serial sections are made through the site of the omnipause neurons, which are thought to be responsible for opsoclonus.<sup>89</sup> In the Lambert-Eaton myasthenic syndrome, electron microscopy reveals binding of antibodies against voltage-gated calcium channels at the presynaptic neuromuscular junction, which disrupts the active sites.<sup>90</sup> Thus, although paraneoplastic syndromes involving the nervous system may all be immune-mediated, the site of damage and the exact mechanism may vary from syndrome to syndrome in ways that are not fully understood.

In paraneoplastic neurologic disorders of the central nervous system, where most of the known target antigens are intracellular proteins, animal models have not provided evidence that antibodies have a role in pathogenesis. Documentation of the expression of major-histocompatibility-complex (MHC) class I and MHC class II antigen-presenting molecules in neurons<sup>91</sup> supports the possibility that T cells recognize intracellular antigen presented to them as an MHC-peptide complex and thereby kill neurons. Identification of antigen-specific T cells in the central nervous system would support this hypothesis, as would an animal model in which antigen-specific T cells mediated neuronal degeneration.

#### TREATMENT

Because paraneoplastic syndromes are considered to be immune-mediated, two treatment approaches have been used: removal of the source of the antigen by treatment of the underlying tumor, and suppression of the immune response. For many paraneoplastic syndromes, the first approach is the only effective treatment.<sup>13,92</sup> In the Lambert-Eaton myasthenic syndrome and myasthenia gravis, plasma exchange or intravenous immune globulin is usually effective in suppressing the immune response.<sup>93</sup> If the disease is mediated by T cells, as is suspected in many central nervous system disorders, such as paraneoplastic cerebellar degeneration with anti-Yo antibodies or encephalomyelitis with anti-Hu antibodies, drugs such as tacrolimus<sup>41</sup> or mycophenolate mofetil<sup>94</sup> may be tried. Because the pathogenesis of many paraneoplastic disorders is unknown and humoral and cell-mediated immunity may both have a role, it may be appropriate to suppress both arms of the immune system.

There are no established protocols for the treatment of most paraneoplastic syndromes, but if the patient's condition is deteriorating, the physician usually uses a combination of either plasma exchange or intravenous immune globulin and immu-

nosuppressive agents such as corticosteroids, cyclophosphamide, or tacrolimus.

There is no established protocol for immunosuppressive treatment. Keime-Guibert and colleagues<sup>95</sup> administered intravenous immune globulin at a dose of 0.5 g per kilogram of body weight per day for five days, intravenous methylprednisolone at 1 g per day for three days, and intravenous cyclophosphamide at 600 mg per square meter of body-surface area for one day on day 4. If there was evidence of improvement or stability, the treatment was repeated three times at three-week intervals. If the patient improved after the third treatment, maintenance treatment with 0.5 g of intravenous immune globulin per kilogram, 1 g of intravenous methylprednisolone, and 600 mg of intravenous cyclophosphamide per square meter was delivered one day monthly for six months.<sup>95</sup> There is less experience with tacrolimus. We have given tacrolimus at a dose of 0.15 mg per kilogram per day for 14 days, followed by 0.3 mg per kilogram per day for 7 days.<sup>41</sup> This regimen decreased the number of activated T cells in the spinal fluid but had no substantial effect on the clinical course.

For most paraneoplastic syndromes, immunotherapy is not effective.<sup>13,95</sup> However, isolated case reports describing responses to various immunotherapeutic interventions encourage physicians to combine immunotherapy with treatment of the cancer in a desperate situation. Since the pathologic features of paraneoplastic neurologic disorders suggest that a destructive immune response is typically present, treatment with immune suppression should begin as expeditiously as possible.

#### PROGNOSIS

Some disorders, such as the Lambert-Eaton myasthenic syndrome and myasthenia gravis, respond well to immunosuppression and subsequently to treatment of the underlying tumor. The peripheral neuropathy associated with osteosclerotic myeloma generally resolves when the tumor is treated with radiotherapy. A few disorders, such as opsoclonus-myoclonus in adults, may respond to treatment of the underlying tumor, immunosuppression, or both, or they may resolve spontaneously. In many instances, it is not clear whether the paraneoplastic syndrome resolves spontaneously or in response to treatment. Disorders involving the central nervous system, such as encephalomyelitis associated with cancer or paraneoplastic cerebellar degeneration,

Table 3. Evidence Supporting the Immune Hypothesis.

Tumor	Nervous System
Neural antigens are present in tumor	Antibodies react with nervous system
Tumors are clinically occult	Antibodies are synthesized intrathecally
Inflammatory (immune) infiltrates are present	Antigen-specific T cells are present in cerebrospinal fluid and brain
Prognosis is better*	Intraneuronal deposits of antibody are present
Spontaneous regression may occur	

\* Patients with paraneoplastic syndromes appear to have a better prognosis with respect to the tumor than do patients with the same type of tumor who do not have paraneoplastic syndromes.

usually respond poorly to treatment, although they may stabilize when the underlying tumor is treated.

The reason for the different prognoses probably has to do with the underlying pathologic features. The Lambert-Eaton myasthenic syndrome and myasthenia gravis are diseases of the neuromuscular junction, which can recover its function once the causal insult has resolved, because there is no loss of the parent neuron. Disorders such as paraneoplastic cerebellar degeneration are usually associated with neuronal loss, and because they evolve subacutely and treatment is often delayed, the neurons die, making recovery impossible. Some central nervous system disorders, such as opsoclonus-myoclonus, may not involve cellular loss and, in fact, may have no identifiable pathologic features. Thus, patients with these disorders, like those with the Lambert-Eaton myasthenic syndrome, have the potential for recovery.

An important question is whether immunosuppression for treatment of the paraneoplastic syndrome stimulates the growth of the tumor. No evidence of this has been reported. Most reports that describe an absence of response of the paraneoplastic syndrome to immunosuppression do not note an exacerbation of the tumor.

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Editor's note: Memorial Sloan-Kettering Cancer Center has licensed patents covering methods used to prepare antigens for assays used in the diagnosis of paraneoplastic syndromes; Drs. Darnell and Posner receive a portion of the royalties.

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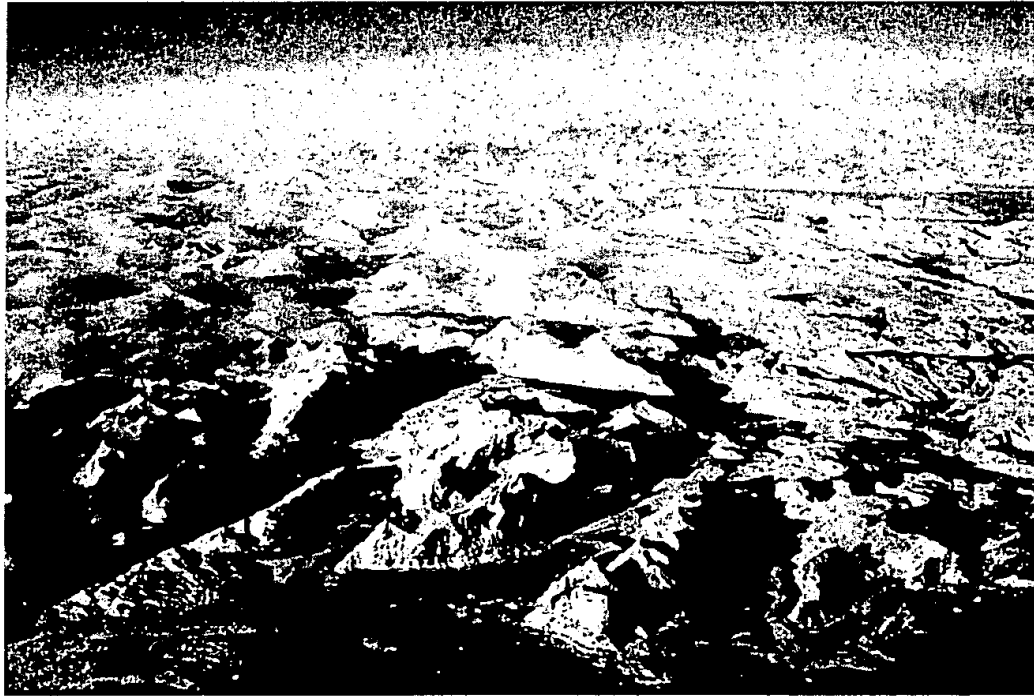
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# Detection and Treatment of Activated T Cells in the Cerebrospinal Fluid of Patients with Paraneoplastic Cerebellar Degeneration

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Patients with paraneoplastic cerebellar degeneration (PCD) offer the opportunity to explore the mechanisms underlying tumor immunity and immune-mediated neuronal degeneration. Cytotoxic T lymphocytes (CTLs) specific for the PCD onconeural antigen cdr2 found in the blood of patients with PCD are likely to be effectors of PCD tumor immunity. Here, we suggest a role for CTLs in the autoimmune destruction of Purkinje neurons. More than 75% of the cells obtained from the cerebrospinal fluid (CSF) of PCD patients were CD3<sup>+</sup> αβ T cells. In patients with active/progressive disease, 20% to 40% of CSF cells were activated T cells, and the CD4<sup>+</sup> helper cells were Th1-type cells. Three PCD patients were given tacrolimus, a specific inhibitor of activated T cells, which markedly reduced these cells in the CSF. Tacrolimus also reduced the number of activated cdr2-specific CTLs in the peripheral blood, but did not lead to tumor recurrence. We suggest that activated cdr2-specific CTLs in the CSF contribute to Purkinje degeneration in PCD, and that tacrolimus therapy may benefit patients with paraneoplastic neurological disease and other T cell-mediated autoimmune neurological disorders.

Albert ML, Austin LM, Darnell RB. Detection and treatment of activated T cells in the cerebrospinal fluid of patients with paraneoplastic cerebellar degeneration. *Ann Neurol* 2000;47:9–17

Paraneoplastic neurological disorders (PNDs) are neuronal degenerations that occur as an indirect result of a malignancy. PNDs arise when systemic tumors aberrantly express proteins normally found only in neurons, and it is believed that the immune system links the clinically evident tumor suppression and neuronal autoimmunity in the disorders. Despite the critical discovery of antibodies specific for tumor and brain (onconeural) antigens (for review, see Posner and Dalmat<sup>1</sup> and Darnell<sup>2</sup>), the pathogenesis of the PNDs remains largely unknown. Although PND antibodies are important for diagnosis, they do not appear to be pathogenic. Treatment to reduce cerebrospinal fluid (CSF) titers of these antibodies are clinically ineffective,<sup>3</sup> and attempts to reproduce the disease in animals by passive antibody transfer or active immunization with onconeural antigens have been unsuccessful.<sup>4,5</sup> Furthermore, PND antigens are either cytoplasmic or nuclear, and it is therefore difficult to understand how antibodies could target tumors or neurons.<sup>2</sup>

Several studies have examined whether T cells play a role in PND pathogenesis. T lymphocytes can be detected in some pathological specimens of brains examined from patients with diffuse PND syndromes,<sup>6–8</sup>

and there is evidence for restricted T-cell receptor (TCR) usage in some patients with Hu antibody-associated PND.<sup>9</sup> In addition, there is an increased incidence of major histocompatibility locus (MHC) class I expression in small-cell lung cancers obtained from Hu<sup>+</sup> PND patients relative to small-cell lung cancers obtained from neurologically normal individuals.<sup>10</sup>

We recently reported that onconeural antigen-specific cytotoxic T lymphocytes (CTLs) are present in the peripheral blood of patients with paraneoplastic cerebellar degeneration (PCD).<sup>11</sup> PCD patients harbor gynecological tumors and a high-titer antibody termed Yo in their serum and CSF. Yo antibodies have been used to clone several cDNAs encoding candidate onconeural antigens,<sup>12–14</sup> one of which, cdr2, is specifically expressed in PCD gynecological tumors.<sup>15</sup> Cytotoxicity assays demonstrated cdr2-specific T cells in 3 of 3 PCD patients.<sup>11</sup> These experiments demonstrated that killer T cells are present, in PCD patients, that recognize the same antigen as the PND antibodies. In contrast to the antibody, these killer T cells offer a mechanism by which the tumor suppression associated with PCD might be mediated.

Here, we extend these observations and provide ev-

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idence that CTLs may play a role in the cerebellar degeneration characteristic in PCD. We found that the CSF of PCD patients harbors activated CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells, and that the latter have a cytokine profile characteristic of a Th1-type cell-mediated immune response. We evaluated the effect of treating these patients with tacrolimus (also known as FK506), a specific inhibitor of activated CTLs that effectively penetrates the blood-brain barrier. Tacrolimus decreased the number of activated T cells in the CSF, and the number of cdr2-specific T cells in the blood, but did not induce tumor recurrence. These studies suggest that CTLs play a pathogenic role in PCD neuronal degeneration, and that tacrolimus may be a safe and effective therapeutic option for PND and other T cell-mediated autoimmune neurological disorders.

## Patients and Methods

### Clinical Studies

All patients were self-referred after a diagnosis of PCD by their primary physicians and were seen at the Rockefeller University Hospital (RUH). Inclusion criteria for our study, as previously described, included confirmation that the patients had PCD.<sup>11</sup>

### Generation of Mononuclear Cell Subsets

Peripheral blood mononuclear cells (PBMCs) were isolated, and purified T cells and dendritic cells were prepared as previously described.<sup>16,17</sup> In brief, T cells were fractionated by rosetting, using neuraminidase (Calbiochem, La Jolla, CA) treated sheep erythrocytes (Colorado Serum, Denver, CO). Dendritic cells were prepared from T cell-depleted fractions by culturing cells for 7 days in granulocyte/macrophage colony-stimulating factor and interleukin (IL)-4, and 4 days in monocyte-conditioned media.<sup>13,14</sup>

### Antibodies

Monoclonal antibodies to the following antigens were used: CD19, CD56, CD3, CD4, CD8,  $\alpha\beta$ -TCR, CD25, interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-2, IL-4, CD14, HLA-DR (Becton Dickinson, San Jose, CA), and CD83 (Coulter Corp, Hialeah, FL). Peripheral blood and CSF cells were phenotyped with this panel of monoclonal antibodies and analyzed on a FACScan (Becton Dickinson). In addition, the DCs prepared from the patients were assayed for phenotypic markers to demonstrate the characteristic CD14<sup>+</sup>, CD83<sup>+</sup>, and HLA-DR<sup>+</sup> phenotype.

### Detection of Intracellular Cytokine Production

Intracellular cytokine profiles were assessed by using a dual-laser FACsCalibur (Becton Dickinson). T-helper cells were first gated by their CD3<sup>+</sup> CD4<sup>+</sup> phenotype, followed by the determination of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-4 concentrations. Cells from CSF and PBMCs were treated with brefeldin A, an inhibitor of secretion, followed by cell fixation and permeabilization, and then intracytoplasmic staining for detection of the accumulated cytokines.<sup>18</sup> As a control, PBMCs

were treated with brefeldin A and cytokine production was stimulated by using phorbol 12-myristate 13-acetate and ionomycin.<sup>18</sup>

### Synthetic Peptides

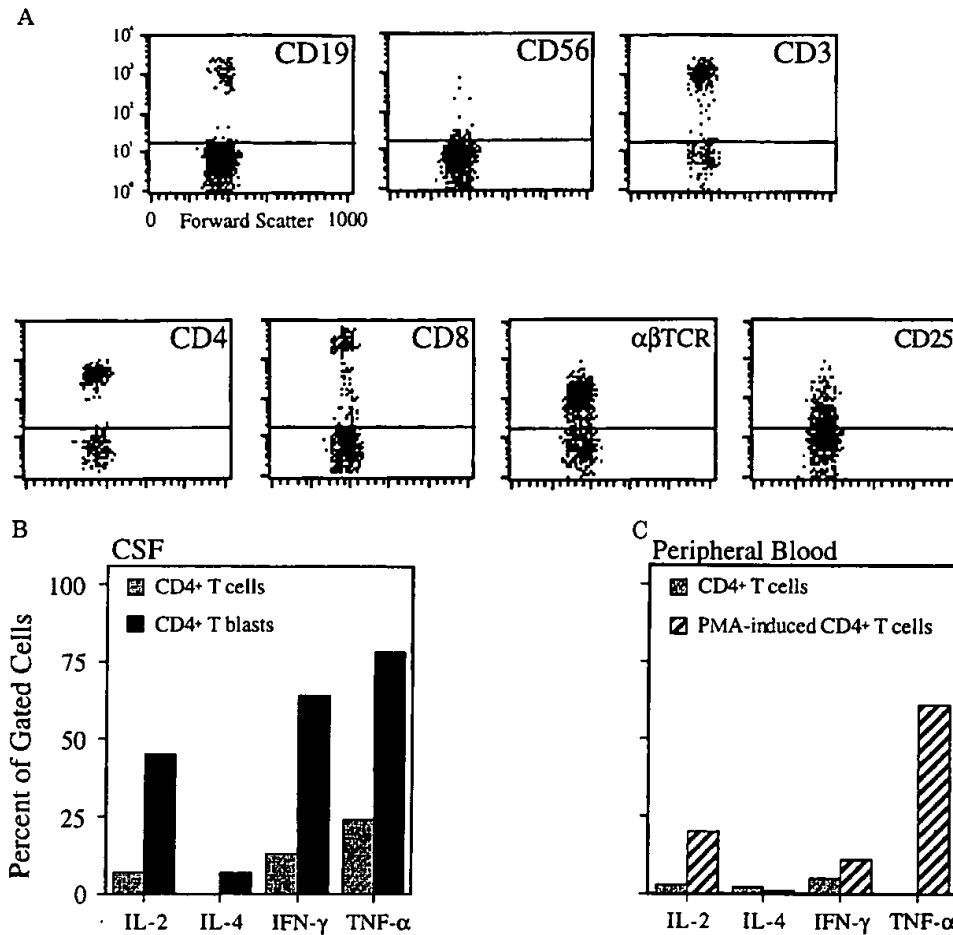
Peptides derived from the human cdr2 gene were predicted for their binding to HLA-A2.1 based on previously defined anchor residues for this well-characterized MHC molecule. These peptides were designated cdr2-1 (KLVPDSLYV amino acids [aa] 273–281, numbering relative to the full-length murine cdr2 sequence,<sup>15</sup> accession no. 1857921), cdr2-2 (SLLEEMFLT aa 289–297), cdr2-3 (QMLQSEHPFV aa 259–268), and cdr2-4 (SLLEEMFLTV aa 289–298), cdr2-5 (TMEEEVGLVL aa 218–227), cdr2-6 (ELEETNQKLV aa 101–110), cdr2-7 (ALKVKYEELL aa 355–364), and cdr2-8 (HLKKTVTMLQ aa 195–205) were synthesized for use in cytotoxicity assays (Biosynthesis Inc, South San Francisco, CA). The HLA-A2.1-restricted immunodominant peptide derived from the influenza matrix protein, designated MP (GILGFVFTL) and a peptide derived from the HuD onco-neural antigen (HuD-3; QFLGPFGAV) served as controls.<sup>19</sup>

### Assays for cdr2-Specific CTLs

T cells were isolated directly from peripheral blood, and the presence of cdr2-specific CTLs was tested by using a conventional Na<sup>51</sup>CrO<sub>4</sub> release assay. T2 cells (a TAP<sup>+/+</sup>, HLA-A2.1<sup>+</sup>, class II<sup>+</sup> cell line) were incubated at room temperature for 1 hour with 1  $\mu$ M concentrations of various peptides, loaded with Na<sup>51</sup>CrO<sub>4</sub>, and used as targets.<sup>11,20</sup> Percent cytotoxicity was determined by using the average values of triplicates from experimental wells (E) compared with average values of spontaneous (S) and total (T) release as follows: % cytotoxicity = [(E – S)/(T – S)]  $\times$  100. Background lysis is shown and ranged from 0% to 3%.<sup>20</sup>

### Patient Histories

PATIENT 9703 (PATIENT 1 IN STUDY BY ALBERT AND COLLEAGUES<sup>11</sup>). A 53-year-old woman was first seen at RUH 18 days after the onset of cerebellar symptoms. Symptoms began with dizziness, and progressed during 5 days to include disabling vertigo, dysarthria, and truncal and appendicular ataxia. Evaluation revealed an enlarged left axillary lymph node, which was found, on biopsy, to contain large tumor cells consistent with adenocarcinoma of the breast. After admission to RUH, the patient had a moderate encephalopathy evident by inattention, and pancerebellar symptoms. Lumbar puncture revealed a CSF pleiocytosis (99 white blood cells/mm<sup>3</sup>) with activated T cells (Fig 1). The patient was treated with tacrolimus (0.15 mg/kg/day) orally for 9 days; peak plasma tacrolimus level was 10.7 ng/ml (expected range, 5–20 ng/ml). Immediately after treatment, there was some improvement in the patient's encephalopathy, which resolved completely thereafter. There was no improvement in the pancerebellar symptoms either immediately



**Fig 1. Activated T cells are detected in the cerebrospinal fluid (CSF) of a patient with acute paraneoplastic cerebellar degeneration (PCD).** (A) CSF cells from Patient 9703 were assayed for expression of the indicated phenotypic markers by FACScan. (B) CSF cells were assayed for their intracellular cytokine profile, using a dual-laser FACSCalibur. T-helper cells were first gated based on their CD3<sup>+</sup> CD4<sup>+</sup> phenotype, and levels of interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-2, and IL-4 were determined. T blasts were selected based on forward scatter and these cells consisted of approximately 10% of the CD3<sup>+</sup> CD4<sup>+</sup> T-cell population. (C) Peripheral blood mononuclear cells (PBMCs) were isolated at the same time and assayed as described. Because there were only background levels of cytokines detected, a positive control was performed by stimulating PBMCs, using phorbol 12-myristate 13-acetate (PMA) and ionomycin to induce Th1-type cytokine production (B).<sup>18</sup> CD56 is a marker for natural killer cells; CD19 is specific for B cells; CD3 is present on all T cells; CD4 and CD8 indicate helper and cytotoxic T-cell subsets, respectively; CD25 is the IL-2 receptor and is a marker for activated T cells.

after treatment or at follow-up 1 year later. The patient underwent a modified radical mastectomy followed by chemotherapy for breast cancer and has subsequently had no evidence of disease (last follow-up, 2 years after tacrolimus).

**PATIENT 9801A (PATIENT 3 IN STUDY BY ALBERT AND COLLEAGUES<sup>11</sup>).** A 64-year-old woman developed abdominal bloating that led to a diagnosis of ovarian cancer in June 1996. She underwent a total abdominal hysterectomy and bilateral salphingo-oophorectomy, which revealed a poorly differentiated papillary serous carcinoma, stage IIIC, with involvement of both ovaries, Fallopian tubes, the uterus, colon, and omentum. She was treated with seven cycles of taxol and

cyclophosphamide. Fourteen months later she developed imbalance, dizziness, and dysarthria that progressed over several weeks and then stabilized.

The patient was first seen at RUH 4 months after the onset of her neurological symptoms, at which time her symptoms were stable. Examination revealed dysarthric but intelligible speech, appendicular ataxia sparing the right side, and gait ataxia requiring ambulation with a walker. Laboratory assays were performed but no treatment was given. Three months later, the patient thought that her cerebellar symptoms were worsening, and she was readmitted to RUH. The patient's ataxia was slightly worse on examination, and she was treated with 0.15 mg/kg/day tacrolimus orally for 14

days. Her CA125 was 10 at completion of therapy, unchanged from her pretherapy level, and there remained no evidence of tumor 1 year after treatment.

**PATIENT 9805.** A 56-year-old woman was admitted to RUH, May 1998, with a diagnosis of PCD and ovarian cancer. The patient presented with slurred speech, difficulty walking, and incoordination 1 year earlier. Approximately 8 months before admission, the patient's serum was found to harbor the Yo antibody. Clinical evaluation revealed an elevated CA125 (58 U/ml), and the patient underwent an exploratory laparotomy that revealed an occult ovarian cancer. She received six cycles of chemotherapy (taxol and carboplatin). Two months before admission, the patient's husband perceived worsening of her neurological function and she was admitted to RUH.

At the time of admission, the patient had moderate cerebellar dysfunction including moderate dysarthria, nystagmus, and appendicular ataxia extremities, and marked truncal ataxia. CSF evaluation revealed activated T cells, and the patient was treated with 0.15 mg/kg/day tacrolimus for 14 days, followed by 0.3 mg/kg/day for 7 days (see below). CA125 was 8 U/ml at the time of admission and was unchanged immediately after treatment and 3 months later.

Seven months after treatment, the patient's gait worsened, and she was readmitted to RUH. The patient's ataxia was slightly worse at the time of examination, and she had developed diplopia. She was treated with 0.2 mg/kg/day tacrolimus for 3 days, followed by 0.1 mg/kg/day tacrolimus for 7 days. Her pretherapy CA125 was 21, and after therapy, her CA125 was 13. There was no evidence of tumor recurrence at 1 year follow-up. A summary of the patients and their T-cell studies are given in the Table.

## Results

### *Detection of Activated T Cells in the CSF of a Patient with Acute PCD*

The first experimental indication of T-cell involvement in PCD neuronal degeneration came from examination of the CSF of an acutely ill patient (Patient 9703). This patient was seen 18 days after the onset of symptoms, at which time lumbar puncture revealed a CSF pleiocytosis. CSF cells were analyzed by fluorescence-

activated cell sorting (FACS) for phenotypic cellular markers. More than 75% of the cells were CD3<sup>+</sup> αβ T cells (see Fig 1A), 40% of which were activated T cells (CD25<sup>+</sup> CD3<sup>+</sup>). Less than 5% of the cells were natural killer cells (CD56<sup>+</sup>), 7% were B cells (CD19<sup>+</sup>), and less than 2% were CD4<sup>-</sup> CD8<sup>-</sup> T cells.

To define the activated cell population, intracellular cytokine staining was performed and cells were assayed by four-color FACS analysis. This revealed helper T cells in the CSF, producing IL-2, IFN-γ, and TNF-α but not IL-4, a cytokine profile characteristic of Th1 cells (see Fig 1B). Moreover, it was possible to demonstrate that the cells producing these cytokines were activated T blasts (based on size). In contrast, CD3<sup>+</sup> CD4<sup>+</sup> cells isolated from the patient's peripheral blood produced little cytokine unless stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (see Fig 1C).

### *Tacrolimus Effectively Eliminated Activated CSF T Cells in the Acute Setting*

Our clinical and CSF analysis of Patient 9703 suggested an acute central nervous system (CNS) process involving activated T cells, and we elected to treat the patient with tacrolimus (FK506), an agent that antagonizes activated T cells and partitions well into the CSF. The patient was treated for 9 days, after which CSF analysis demonstrated a remarkable decrease in the number of activated T cells (30% of the total cells in the CSF before treatment, to <1% after treatment; Fig 2A).

### *Tacrolimus Effectively Reduced the Number of Activated CSF T Cells in Chronic PCD*

It was also possible to detect activated T cells in the CSF of PCD Patient 9805 who had developed neurological symptoms 8 months earlier, and who presented with progressive disease symptoms. FACS analysis revealed the presence of activated T cells in the CSF (see

*Table. Subjects with Paraneoplastic Cerebellar Degeneration Treated with Tacrolimus Therapy*

Patient	Tumor	cdr2-Specific CTLs		Activated T Cells in CSF (cells/ml) <sup>a</sup>	
		Activated	Memory	Before Therapy	After Therapy
Patient 9703	Breast	Yes	Yes	3.0 × 10 <sup>4</sup>	<660
2nd admission		No	ND	ND	ND
Patient 9801a	Ovarian	No	Yes	IM	ND
2nd admission		Yes	ND	IM	ND
Patient 9805	Ovarian	Yes	Yes	1.2 × 10 <sup>3</sup>	330
2nd admission		ND	Pending	5.0 × 10 <sup>3</sup>	1.2 × 10 <sup>3</sup>

<sup>a</sup>As defined by CD3<sup>+</sup> CD25<sup>+</sup> surface labeling.

CTL = cytotoxic T lymphocyte; ND = not done; IM = insufficient material for fluorescence-activated cell sorting analysis.

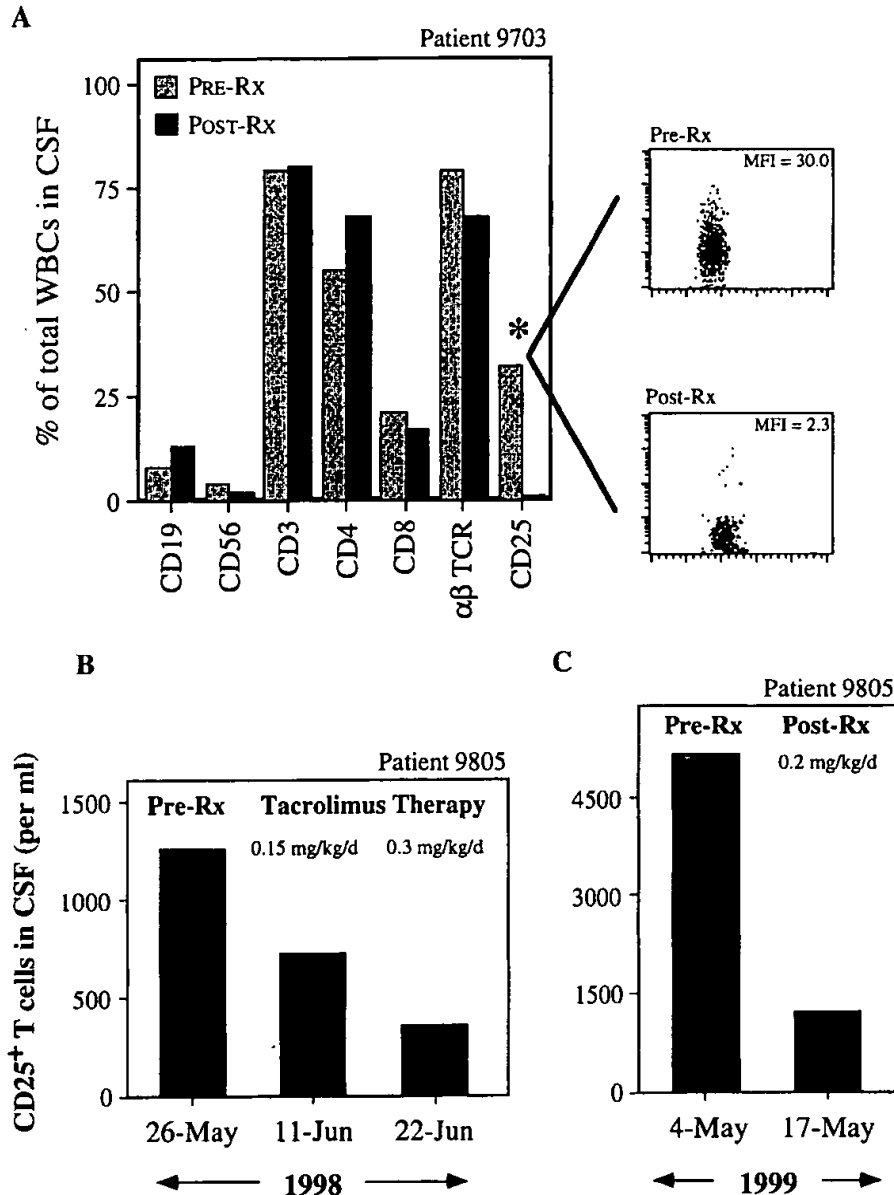


Fig 2. Activated T cells detected in the cerebrospinal fluid (CSF) of paraneoplastic cerebellar degeneration (PCD) patients are eliminated by treatment with tacrolimus. (A) Activated CD25<sup>+</sup> T cells are present in the CSF obtained from a patient presenting with acute PCD. Actual dot plots for staining with anti-CD25 are shown to the right of the bar graph. Contaminating red blood cells were excluded by forward and side scatter properties.  $p < 0.0001$ , by McNemar  $\chi^2$  analysis. \* = significant change in the number of activated T cells after tacrolimus therapy; WBCs = white blood cells; MFI = mean fluorescence intensity. (B) Activated CD25<sup>+</sup> T cells are present in the CSF of a patient with chronic PCD. After tacrolimus treatment at the indicated doses, the absolute number of activated T cells decreased in a dose-dependent manner. (C) At 1-year follow-up in the same patient as B, activated CD25<sup>+</sup> T cells were again present in the CSF in high numbers, which was consistent with worsening neurological symptoms. Again, these activated T cells in the CSF were reduced after tacrolimus treatment.

Fig 2B). The patient was treated with tacrolimus, 0.15 mg/kg/day for 14 days, after which activated CSF T cells were reduced in number but still detectable (9 white blood cells/mm<sup>3</sup>, with 8% of the population being CD25<sup>+</sup> T cells). A higher dose of tacrolimus (0.3 mg/kg/day) was given. During this time, the patient

developed transient peripheral neuropathic symptoms (mild diplopia and titubation), and therapy was discontinued after 7 days. Repeat CSF analysis revealed 3 white blood cells/mm<sup>3</sup>, approximately 11% of which were activated CD25<sup>+</sup> T cells, a decrease of 73% from the start of the therapeutic protocol. The side effects of

the tacrolimus resolved by 2 days after treatment. These studies demonstrate that tacrolimus can mediate a dose-dependent decrease in the absolute number of activated T cells.

Patient 9805 remained neurologically stable after treatment for 7 months and then developed worsening of her cerebellar symptoms. After readmission, CSF analysis revealed a reaccumulation of activated T cells. The patient was treated with a relatively low-dose tacrolimus, which was well tolerated and reduced the number of activated T cells in her CSF by approximately fivefold (see Fig 2C).

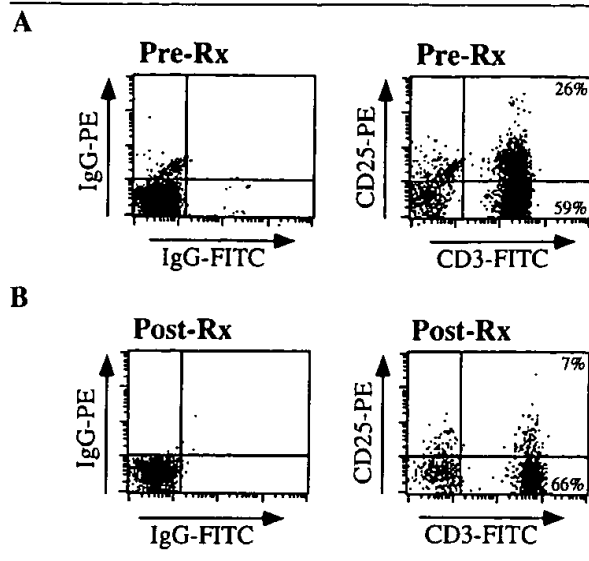
#### *Tacrolimus Reduced the Number of Activated T Cell in the Peripheral Blood*

Our studies suggest a means by which activated T cells in the CSF of patients with PCD may be eliminated, and, if given early in the course of disease, could help prevent further neurodegeneration. However, an important consideration in such treatment is the effect of tacrolimus on activated T cells in the peripheral blood, as this is likely the source of this effector cell population in the CSF.

This issue was particularly relevant in Patient 9801a because, although she had no detectable cells in her CSF, activated T cells in the peripheral blood correlated with a worsening of her neurological condition. During the patient's first RUH admission, when she was neurologically stable, there were no detectable activated T cells in her peripheral blood (data not shown). However, when she was readmitted to RUH with worsening cerebellar symptoms, more than 30% of the T cells in her peripheral blood expressed activation markers (Fig 3A). After a 14-day course of tacrolimus, the percentage of peripheral blood CD25<sup>+</sup> CD3<sup>+</sup> T cells decreased threefold (see Fig 3B).

#### *Tacrolimus Eliminated cdr2-Specific CTLs in the Peripheral Blood of PCD Patients*

PCD Patient 9805 had a history of ovarian cancer, moderate cerebellar dysfunction, and no clinical evidence of oncological disease. As discussed, CSF analysis revealed CD25<sup>+</sup> T cells by FACS analysis (see Fig 2B), and the patient was treated with tacrolimus for 14 days. To evaluate the effect of treatment on cdr2-specific CTLs, cytotoxicity assays of activated cdr2-specific T cells were performed before and after treatment (Fig 4; and data not shown). Activated CD8<sup>+</sup> CTLs were assayed directly from whole blood by testing their ability to lyse cdr2 peptide pulsed target cells. Before the initiation of tacrolimus therapy, activated CTLs specific for target cells presenting cdr2 peptides were detected. These results confirm the presence of activated cdr2-specific CTLs among PCD blood cells,<sup>11</sup> and demonstrate that activated cdr2-specific CTLs may be detected in the blood of PCD patients



**Fig 3.** Decreased numbers of activated T cells in the peripheral blood after treatment with tacrolimus. (A) Fluorescence-activated cell sorting analysis of a T cell-enriched fraction of peripheral blood mononuclear cells (PBMCs) obtained from Patient 9801a before tacrolimus treatment. Anti-CD3 was used to identify the T cells and anti-CD25 to identify the activated cells; >30% of the T cells circulating in the peripheral blood were activated cells. (B). One day after completion of tacrolimus treatment, peripheral blood was tested for CD25<sup>+</sup> CD3<sup>+</sup> T cells, revealing a threefold reduction in activated cells.  $p < 0.0001$ , by McNemar  $\chi^2$  analysis. PE = phycoerythrin; FITC = fluorescein isothiocyanate.

even in the absence of overt tumor. Repeat analysis immediately after tacrolimus treatment revealed that activated cdr2-specific CTLs could no longer be detected.

#### **Discussion**

##### *Pathogenesis of PCD*

We have studied the cellular immune response in patients with PCD, providing the first phenotypic description of inflammatory cells in the CSF of acute and chronically ill patients. Although the Yo antibody present in PCD patients has provided a crucial marker for diagnosis and for understanding the nature of the antigenic target, the antibody has had an uncertain role in disease pathogenesis. Cloning of the full-length cdr2 antigen<sup>15</sup> confirmed immunohistochemical observations that the target antigen is intracellular,<sup>21,22</sup> by revealing that the cDNA encodes no signal sequence or transmembrane domain. Although there is some evidence that neurons, including cerebellar Purkinje neurons, may nonspecifically take up antibodies in animals,<sup>23-25</sup> such experiments have failed to reproduce disease, and interest has turned toward whether a cellular immune response plays a role in PCD. Examination of peripheral blood has revealed the presence of



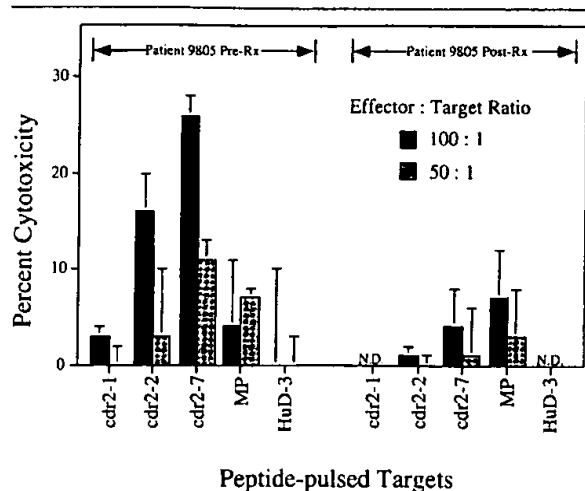


Fig 4. Activated *cdr2*-specific T cells in the peripheral blood of a paraneoplastic cerebellar degeneration (PCD) patient are reduced after tacrolimus treatment. T cells were isolated from the peripheral blood of Patient 9805 during her first admission to Rockefeller University Hospital. These were used directly as effectors in a standard  $^{51}\text{Cr}$  release assay by using peptide-pulsed T2 cells as targets. *cdr2*-specific cytotoxic T lymphocytes were detected with specificity for the *cdr2*-7 peptide and, to a lesser extent, the *cdr2*-2 peptide. Both responses were titratable and both were specific to the patient, because a normal HLA-A2.1<sup>+</sup> individual was tested at the same time and no *cdr2*-specific cells were detected (data not shown). After 14 days of tacrolimus, T cells were again isolated from the patient and tested for their ability to lyse peptide-pulsed T2 cells. Less than 5% cytotoxicity was observed for the *cdr2*-7 and *cdr2*-2 peptide-pulsed targets. Negative controls in this experiment included the use of irrelevant peptides pulsed onto T2 cells. HuD-3 is an irrelevant control peptide, and MP is the HLA-A2.1 immunodominant epitope derived from the influenza matrix protein. Data shown represent mean values from triplicate wells, and error bars indicate SD. N.D. = not done.

*cdr2*-specific CTLs in 5 of 5 PCD patients (Albert and associates,<sup>11</sup> this study, and unpublished data), demonstrating antigen-specific T cells that are likely to mediate the tumor suppression evident in PCD.

Within the CNS, a role for cellular immunity in disease pathogenesis in PND has been suggested by reports of T cells present in inflammatory infiltrates in pathological specimens.<sup>26</sup> We find that most cells in the CSF of both acute and chronically ill PCD patients are classic  $\alpha\beta$  T cells. In the acute setting, which provides perhaps the clearest indication of disease pathogenesis, not only are the T cells abundant, but a large percentage of these cells (40%) are activated (see Fig 1), indicating the presence of an ongoing T cell-mediated immune response. Moreover, the cytokine profile of the CD4<sup>+</sup> cells present revealed that these cells were of the Th1 type.<sup>27,28</sup> The Th1 cytokine profile (IFN- $\gamma$ , TNF- $\alpha$ , and IL-2) of lymphocytes within the CSF sup-

ports that PCD patients have an active cell-mediated autoimmune response ongoing within their CNS.

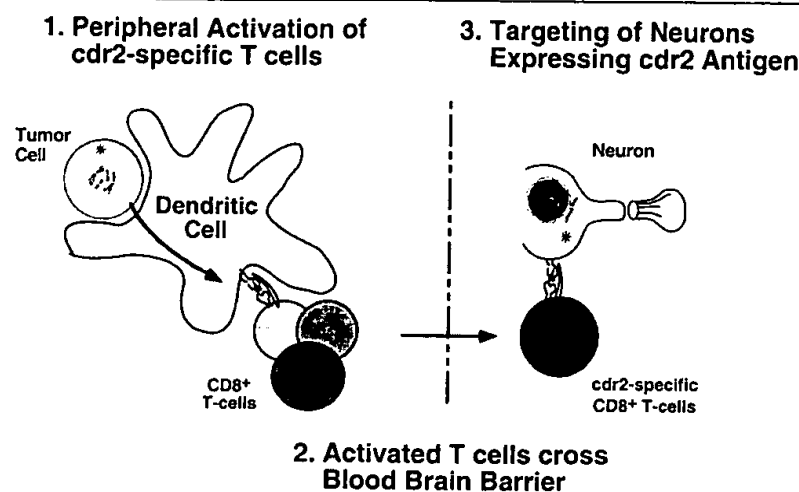
Taken together, our results strongly support that antigen-specific CTLs play a pathogenic role in PCD. Although we would suppose that *cdr2*-specific CTLs are the effectors responsible for mediating neuronal degeneration in addition to tumor immunity, we have not been able to demonstrate this directly, and future work will need to develop assays with which such cells in CSF can be detected.

#### Treatment of PCD

Because our data suggest a role for activated T cells in Purkinje neuronal degeneration, we have begun to explore treatment directed at eliminating these cells from the CSF. Previous treatments to reduce antibody titers, including plasmapheresis, which reduces intrathecal antibody titers, azathioprine, and steroids, have been clinically ineffective. We chose tacrolimus (FK506) for its high lipid partition coefficient, favoring entry into the CNS, and because its mechanism of action is directed against activated T cells. Tacrolimus binds a cellular protein, FKBP12, forming a complex that inhibits calcineurin. This alters the phosphorylation of the transcription factor NF-ATc, preventing its transport to the nucleus and the induction of genes involved in T-cell activation (eg, IL-2 and CD25). In addition, FKBP12 binds to the transforming growth factor- $\beta$  (TGF- $\beta$ ) receptor, serving as a docking protein for calcineurin and inhibiting TGF- $\beta$  signaling. By inhibiting FKBP12, tacrolimus enhances the action of TGF- $\beta$ , a potent suppressor of Th1-type T-cell responses. Thus, tacrolimus acts via multiple pathways to suppress precisely the types of T-cell responses we found in the CSF of PCD patients.

The clinical evidence of tumor suppression in PCD, and our demonstration of *cdr2*-specific CTLs (Albert and associates<sup>11</sup>; see Fig 4), suggest that tacrolimus treatment could exacerbate patients' underlying tumors by suppressing tumor immunity. In both short-term and long-term follow-up, there has been no recurrence of clinically evident tumor in the 3 patients we have treated, despite our finding that tacrolimus decreased peripheral blood-activated T cells. In fact, blocking T cells in the periphery may have some benefit; activation of peripheral T cells may induce adhesion molecules such as VCAM and ICAM, which may be an important prerequisite to T cells crossing the blood-brain barrier.<sup>29</sup> Taken together, our data indicate that tacrolimus may be given safely in short courses in PND patients without triggering a tumor recurrence.

Tacrolimus was well tolerated neurologically in our study. One patient experienced numbness at a high dose, and careful observation is warranted, including care distinguishing neurological worsening from disease progression versus drug effect. Although tacrolimus



*Fig 5. Model for the pathogenesis of paraneoplastic cerebellar degeneration (PCD). PCD is suggested to be initiated when breast or ovarian tumors express cdr2, a protein that is normally made only in neurons. In part because of the immunologically privileged state of neurons and neuronal antigens, the immune system recognizes the neuronal protein as foreign when ectopically expressed in tumor cells. A cdr2-specific T-cell response is generated, which is associated with effective tumor immunity. Once activated, these T cells may cross the blood-brain barrier and become effectors responsible for recognizing and killing neurons that normally express the cdr2 onconeural antigen.*

may stabilize the progression of neurological disease, we cannot draw any conclusion from this study, given the small sample size, the lack of control patients, and difficulty with defining the natural course of the disease. For example, PCD patients may have progressive symptoms during a period ranging from 4 days to at least 6 months.<sup>30</sup>

#### *The Pathogenesis of Neuronal Degeneration in Paraneoplastic Neurological Disease*

Our data suggest common features in the pathogenesis and potential treatment of PNDs and other autoimmune neurological disorders. Indeed, our current model for PND disease pathogenesis (Fig 5) bears some similarity to the existing model for the pathogenesis of multiple sclerosis.<sup>31</sup> In both disorders, the initiating event is proposed to be the activation of brain-specific T cells outside of the nervous system. In PCD, our data indicate that cdr2-expressing tumor cells initiate the immune responses, presumably because the tumor cells are expressing what is perceived to be a "foreign" (neuron-specific) intracellular protein. In multiple sclerosis, an analogous (perhaps infectious) systemic trigger has been proposed to result in the peripheral activation of myelin-reactive T cells. Once activated, brain-specific T cells cross the blood-brain barrier where they may now target the Purkinje neurons or myelin sheath, respectively. Although it has been generally believed that neurons do not express self-antigen on MHC class I molecules, recent work has identified MHC I expression in neurons,<sup>32</sup> including cerebellar Purkinje neurons, suggesting a means by which the T cells identified here could mediate neuronal damage.<sup>33</sup> Clearly, further characterization of the nature of the autoimmune response within the CNS

in the PNDs will be important in considering the pathogenesis and treatment of autoimmune neurological disorders.

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